

Release of protein A from the cell wall of *Staphylococcus aureus*

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Staphylococcal protein A (SpA) is anchored to the cell wall envelope of *Staphylococcus aureus* by sortase A, which links the threonyl (T) of its C-terminal LPXTG motif to peptidoglycan cross-bridges (i.e., Gly₅). SpA binds the Fc γ domains of IgG and protects staphylococci from opsonophagocytic clearance. Moreover, SpA cross-links B-cell receptors to modify host adaptive immune responses. The mechanisms whereby SpA is released from the bacterial surface to access the host's immune system are not known. Here we demonstrate that SpA is released with murein tetrapeptide-tetraglycyl [L-Ala-D-iGln-(SpA-Gly₅)L-Lys-D-Ala-Gly₄] linked to its C-terminal threonyl. LytN, a cross-wall murein hydrolase, contributes to the release of SpA by removing amino sugars [i.e., N-acetylmuramic acid-N-acetylglucosamine (MurNAc-GlcNAc)] from attached peptidoglycan, whereas LytM, a pentaglycyl-endopeptidase, triggers polypeptide release from the bacterial envelope. A model is proposed whereby murein hydrolases cleave the anchor structure of released SpA to modify host immune responses.

surface protein | Gram-positive bacteria | sortase-anchored protein

The Gram-positive bacterium *Staphylococcus aureus* is a pathogen of humans (1). Cells of *S. aureus* are surrounded by a thick layer of highly cross-linked cell wall peptidoglycan (2). The peptidoglycan layer is formed from lipid II precursors, C₅₅-(PO₃)₂-N-acetylmuramic acid (MurNAc)-(L-Ala-D-iGln-(Gly₅)L-Lys-D-Ala-D-Ala)-GlcNAc (3), via the transpeptidation and transglycosylation reactions of cell wall synthesis to generate [MurNAc-(L-Ala-D-iGln-(Gly₅)L-Lys-D-Ala)-GlcNAc]_n polymer (4). Assembled peptidoglycan is a single large macromolecule that protects bacteria against osmotic lysis (5) and also functions as scaffold for the anchoring of wall teichoic acids (6) and proteins (7). These secondary cell wall polymers promote specific interactions between staphylococci and host tissues (8). Cell wall-anchored surface proteins are synthesized as precursors with N-terminal signal peptides and C-terminal LPXTG motif sorting signals (9). Following cleavage of the N-terminal signal peptide by signal peptidase, the C-terminal sorting signal is cleaved by sortase A between the threonyl (T) and the glycyl (G) of the LPXTG motif (10). Sortase A forms an acyl enzyme, capturing the C-terminal carboxyl group of cleaved surface proteins with its active site cysteine thiol (11). These acyl intermediates are relieved by the nucleophilic attack of the amino group of pentaglycyl within lipid II and incorporated into the cell wall via the transpeptidation and transglycosylation reactions (7, 12).

The genomes of *S. aureus* isolates harbor 17 to 22 genes encoding LPXTG motif surface proteins, which can be further classified as precursors with canonical or YSIRK-G/S signal peptides (13). Surface proteins with canonical signal peptides are secreted and immobilized to peptidoglycan near the cell poles of dividing staphylococci (14). In contrast, precursors with YSIRK-G/S signal peptides are secreted into the cross-wall, a membrane enclosed compartment for the de novo synthesis of peptidoglycan that separates daughter cells during division (14). When precursors with YSIRK-G/S signal peptides and LPXTG motif sorting signals have been deposited at the cross-wall and its peptidoglycan has been split, surface proteins are displayed over the staphylococcal surface (14). YSIRK-G/S precursors include proteins with important virulence functions that are synthesized

in large abundance, including clumping factor A (15), fibronectin binding proteins (16, 17), iron-regulated surface protein B (18), and staphylococcal protein A (SpA) (19).

SpA binds human or animal Ig via its Ig-binding domains that capture the Fc γ domain of IgG or the Fab domain of V_H3-clan IgG and IgM antibodies (20, 21). SpA binding to the Fc γ domain blocks the ability of antibodies with specific binding activities for the staphylococcal surface to promote Fc receptor-mediated opsonophagocytosis and bacterial killing (22). SpA binding to the Fab domain of V_H3-clan IgM triggers B-cell receptor cross-linking and clonal expansion of B lymphocytes, which eventually undergo apoptotic collapse (23). During infection, this B-cell superantigen activity of SpA ablates host adaptive immune responses against many staphylococcal antigens (24). Although *S. aureus* disease predominantly manifests as localized skin or soft-tissue infection, its suppressive effects on the immune system appear to be general (25). If so, we wondered whether SpA, a key factor for staphylococcal immune evasion, is released from the bacterial surface.

Results

Protein A Release from *S. aureus*. To examine the fate of protein A by immunoblotting, *S. aureus* Newman, as well as its isogenic *spa*, *sbi*, and *spa/sbi* mutants, were grown as tryptic soy broth (TSB) cultures and centrifuged, and extracellular medium was separated from the bacterial sediment. *S. aureus sbi* encodes staphylococcal binder of Ig, a secreted polypeptide that binds the Fc γ domain of IgG, similar to protein A (*spa* encoded) (26). Proteins in both fractions, extracellular medium and the bacterial sediment, were precipitated with trichloroacetic acid (TCA). Proteins tethered to the envelope were released with lysostaphin (27), and samples were analyzed by immunoblotting with SpA_{KKAA}-mAb 3F6 (28). *S. aureus* Newman cultures harbored 84.90% (± 1.28) of SpA in the bacterial envelope, whereas 15.10% (± 1.28) were released

Significance

Surface proteins are linked to the cell wall of Gram-positive bacterial pathogens by a mechanism requiring LPXTG motif sorting signals and sortase. Here we show that surface proteins are also released from the bacterial surface into the extracellular milieu and may fulfill functions similar to secreted polypeptides. We demonstrate that protein A of *Staphylococcus aureus*, a B cell superantigen, is released with peptidoglycan linked to its C terminus. Release of protein A involves murein hydrolases that remove immunostimulatory N-acetylmuramic acid and GlcNAc residues and liberate the polypeptide from the envelope. This mechanism of surface protein release may be universal for surface proteins of Gram-positive bacteria.

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into the extracellular medium (Fig. 1A). As controls, the *spa* and *spa/sbi* mutants did not express *spa*. The *sbi* variant was not affected for *spa* expression, SpA anchoring to the cell wall, or SpA release into the extracellular medium (Fig. 1A). SrtA, the membrane-anchored transpeptidase (29), was not released into the extracellular medium (Fig. 1A).

Following ribosomal synthesis, SpA precursors are secreted within 2 min across the membrane and processed by sortase A (9). Are SpA molecules initially anchored to the cell wall and subsequently released into the medium, or do staphylococci steadily release some portion of protein A? To address this, *S. aureus* Newman was pulse-labeled with [³⁵S]methionine/cysteine. At timed intervals, culture aliquots were precipitated with TCA, suspended in buffer, and split into two samples. One sample was treated with hot SDS to solubilize proteins released into the medium; the other sample was treated with lysostaphin to solubilize anchored protein A (30). Immediately following the chase (0 min), and 30 min thereafter, all pulse-labeled protein A required lysostaphin treatment to gain solubility (Fig. 1B). Beginning at 60 min, 1.46% of protein A was found soluble without lysostaphin treatment, and 15.2% of pulse-labeled SpA was released at 120 min (Fig. 1B). Sortase A served as loading control and was not solubilized without lysostaphin treatment (Fig. 1B). Taken together, these data indicate that SpA precursors are first anchored to the cell wall envelope and subsequently released into the extracellular medium.

Structure of Released Protein A. SpA was purified from the culture medium of *S. aureus* Newman lacking *sbi* by affinity chromatography on human IgG-Sepharose (31). Edman degradation released amino acid residues of protein A that matched the N-terminal sequence of mature SpA (32). The first Edman cycle released fourfold higher molar amounts of amino acid, i.e., alanine, the N-terminal residue of SpA and a constituent of peptidoglycan, than the next cycle (glutamine). These data suggest

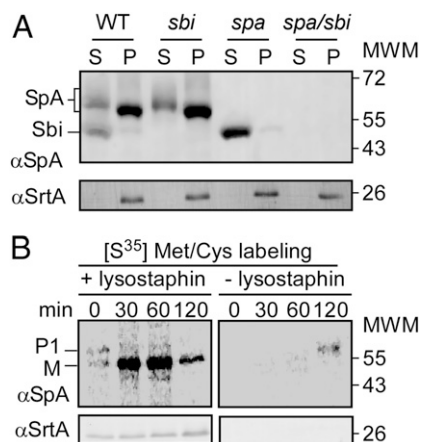


Fig. 1. *S. aureus* releases protein A from the cell wall envelope. (A) *S. aureus* Newman WT or *sbi*, *spa*, or *spa/sbi* cultures were centrifuged and the extracellular medium separated with the supernatant (marked as "S") from the bacterial pellet (marked as "P"). After treatment of the staphylococcal cell wall envelope with lysostaphin, proteins in both fractions were analyzed by immunoblotting with polyclonal antibodies against protein A (α SpA) or sortase A (α SrtA). (B) *S. aureus* Newman cells were washed in PBS solution, pulse-labeled with [³⁵S]Met/Cys, and mixed with fresh culture media. At timed intervals after labeling (0, 30, 60, 120 min), two aliquots from the culture were precipitated with TCA. One sample was treated with lysostaphin to release sortase (cell wall)-anchored proteins (Left), whereas the other was treated with hot SDS to solubilize released protein A molecules (Right). Radioactive samples were immunoprecipitated with α SpA and analyzed by SDS/PAGE and PhosphorImager. Immunoblotting with α SrtA was used as fractionation control.

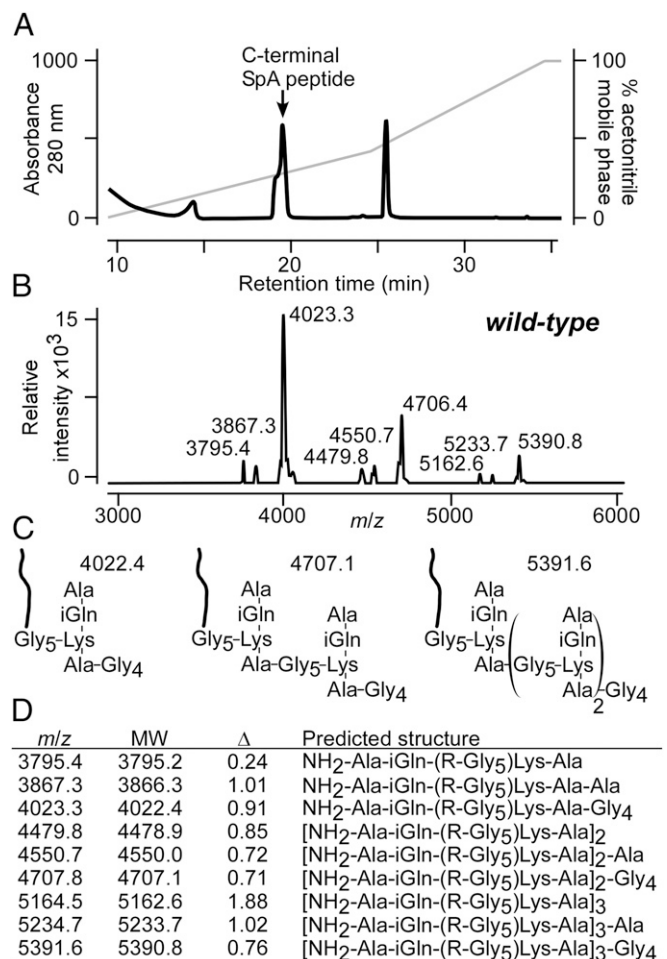


Fig. 2. Structure of released protein A. (A) Protein A was purified by affinity chromatography from the culture medium and cleaved with cyanogen bromide, and C-terminal peptides were isolated by RP-HPLC and identified by Edman degradation (Table S2). (B) C-terminal SpA peptides were subjected to MALDI-TOF MS and ion signals were recorded. (C) Model for the predominant ions signals generated by C-terminal SpA peptides. (D) Observed and predicted (MW) *m/z* and differentials (Δ) for SpA peptides released by WT *S. aureus* with their predicted structures.

that phenylthiohydantoin cleaved alanine from the N terminus of SpA and from wall peptides linked to its C terminus [NH₂-Ala-iGln-(Gly₅)-Lys-Ala-COOH; Table S1].

Purified protein A molecules were treated with cyanogen bromide, which cleaves polypeptide chains at methionyl residues. Peptide fragments were purified by RP-HPLC and analyzed by Edman degradation. The absorbance peak at 19 min yielded the sequence NH₂-IKPGQ consistent with the C-terminal fragment of protein A (NH₂-IKPGQELVVDKKOPANHADANKAQALPET; Fig. 2A). MALDI-TOF MS identified ion signal *m/z* as 4,023.35, 4,706.46, and 5,390.89 (Fig. 2B). These data cannot be explained on the basis of the predicted protein sequence but must be accounted for by the mass of protein A plus the cell wall component to which it is linked. We therefore built models for protein A linked to peptidoglycan fragments and interpreted *m/z* 4,023.35 as released SpA (R = NH₂-IKPGQELVVDKKOPANHADANKAQALPET) linked to the pentaglycyl cross-bridge (Gly₅) of a peptidoglycan tetrapeptide-tetraglycine [NH₂-Ala-iGln-(R-Gly₅)-Lys-Ala-Gly₄; Fig. 2C]. Compounds *m/z* 4,706.46 and 5,390.89 were interpreted as SpA tethered to cross-linked peptidoglycan with the same structure [NH₂-Ala-iGln-(R-Gly₅)-Lys-Ala-Gly₄]₂₋₃; Fig. 2C. Minor ion signals were interpreted as SpA tethered to higher cross-

linked wall peptides [NH₂-Ala-iGln-(R-Gly₅)-Lys-Ala-Gy₄]_n or to peptidoglycan with cell wall pentapeptides NH₂-Ala-iGln-(R-Gly₅)-Lys-Ala-Ala, respectively (Fig. 2 C and D).

Protein A Released from *S. aureus atl*, *sle1*, and *lytN* Mutants. The structure of released SpA may be shaped by muralytic enzymes that split the cross-wall compartment during staphylococcal growth, i.e., Atl [*N*-acetylmuramoyl-L-alanine amidase (amidase) and glucosaminidase] (33, 34), Sle1 (CHAP domain amidase and D-Ala-Gly endopeptidase) (35), and LytN (CHAP domain amidase and D-Ala-Gly endopeptidase) (36, 37) (Fig. 3A). If so, *S. aureus* Newman mutants lacking the structural genes for these muralytic enzymes may release protein A with altered peptidoglycan structure. This was tested by purifying SpA from the medium of cultures inoculated with the *S. aureus* Newman *atl* variant that also lacks *sbi* (38). Following cyanogen bromide cleavage and RP-HPLC purification, protein A peptides from the *atl* mutant were subjected to MALDI-TOF MS, which identified ion signals with *m/z* 3,339.86 and 3,796.12 (Fig. 3B). Structural models for released peptide linked to tetraglycyl (R-Gly₄) and peptidoglycan tetrapeptide [NH₂-Ala-iGln-(R-Gly₅)-Lys-Ala] fit the observed mass signals (Fig. 3B and C).

Sle1 is secreted into medium and subsequently binds to the cross-wall section of peptidoglycan via its LysM domains, connecting with glycan strands (MurNAc-GlcNAc)_n that have not been decorated with wall teichoic acids (polyribitol-phosphate) at the C6-hydroxyl of MurNAc (37). LytN precursor is secreted via its YSIRK-G/S motif signal peptide into the cross-wall, and its CHAP domain cleaves peptidoglycan through its amidase and endopeptidase activities (36). Staphylococcal *sle1* (35) and *lytN* mutants display defects in cell separation with inadequate splitting of cross-wall peptidoglycan (35, 36). Protein A was purified from the extracellular medium of *S. aureus* Newman *sle1* and *lytN* mutants (also lacking *sbi*) and cleaved with cyanogen bromide, and C-terminal peptides were analyzed by MS. SpA released from the *sle1* mutants generated the ion signals *m/z* 3,865.17 and 4,023.24, which were interpreted as NH₂-Ala-iGln-(R-Gly₅)-Lys-Ala-Ala and NH₂-Ala-iGln-(R-Gly₅)-Lys-Ala-Gly₄ (Fig. 4A and C). We also detected peptidoglycan with the similar structure and two to four cross-links [NH₂-Ala-iGln-(R-Gly₅)-Lys-Ala]₂₋₄-Ala (Fig. 4A and C). SpA peptides released by the *lytN* mutant

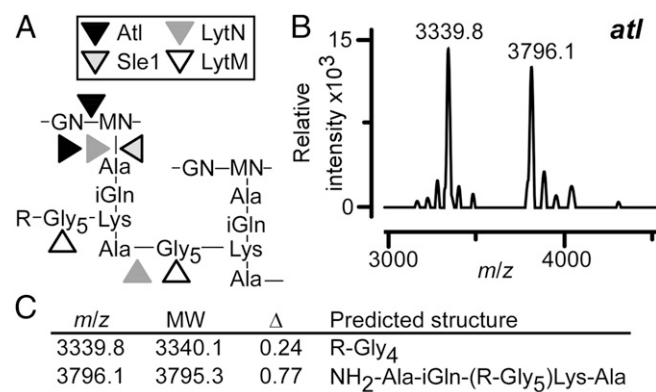


Fig. 3. *S. aureus* murein hydrolases impact the release of protein A. (A) Structure of *S. aureus* peptidoglycan comprised of the repeating disaccharide *N*-acetylglucosamine-(β 1-4)-*N*-acetylmuramic acid (GN-MN) with linked wall peptide (Ala-iGln-Lys-Ala) and pentaglycine cross-bridge (Gly₅). Arrowheads identify the cleavage sites of murein hydrolases that act at the cross-wall (Atl, Sle1, and LytN) or that function as glycylo-endopeptidases (LytM). (B) MALDI-TOF MS of C-terminal SpA peptides released from the *S. aureus atl* mutant. (C) Observed and predicted (MW) *m/z* ratios and their differentials (Δ) for SpA peptides released by the *S. aureus atl* mutant with their predicted structures.

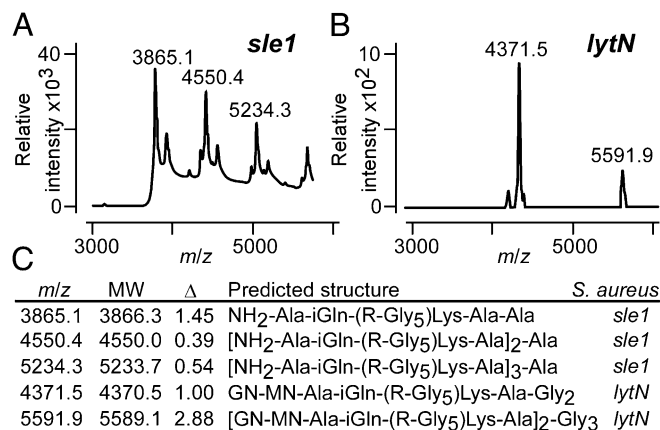


Fig. 4. Cross-wall murein hydrolases shape the structure of released protein A. (A) MALDI-TOF mass spectrometry of C-terminal SpA peptides released from the *S. aureus sle1* (A) and *lytN* (B) mutants. (C) Observed (*m/z*) and predicted (MW) mass-to-charge ratios and their differentials (Δ) for SpA peptides released by the *S. aureus sle1* and *lytN* mutants with their predicted structures.

generated the ion signals *m/z* 4,371.57 and 5,591.98, which were interpreted as MurNAc[Ala-iGln-(R-Gly₅)-Lys-Ala-Gly₂]-GlcNAc as well as [MurNAc(Ala-iGln-[R-Gly₅]-Lys-Ala)-GlcNAc]₂-Gly₃ (Fig. 5B and C). These data suggest that LytN is responsible for cleaving the amide bonds between MurNAc and wall peptides, which enables the release of protein A with cross-linked peptidoglycan that lacks the amino sugars of the cell wall.

LytM Releases Protein A from the *S. aureus* Envelope. LytM is a secreted glycylo-glycine endopeptidase of *S. aureus* (39), and we wondered whether this enzyme may be involved in the release of protein A. Unlike Atl, Sle1, and LytN, which are targeted to the cross-wall, the subcellular location of LytM is not yet known. SpA peptides released by the *lytM* mutant were not abundant. Nevertheless, these peptides generated *m/z* 3,795.87, 3,866.87, and 4,022.94, i.e., the same structures as WT *S. aureus*: Ala-iGln-(R-Gly₅)-Lys-Ala, Ala-iGln-(R-Gly₅)-Lys-Ala-Ala, and Ala-iGln-(R-Gly₅)-Lys-Ala-Gly₄ (Fig. S1).

To quantify the release of SpA, *S. aureus* strains were grown to similar densities and protein A release was analyzed by immunoblotting. Cultures were centrifuged, and staphylococci were treated with lysostaphin to determine the total amount of SpA associated with the envelope. The supernatant was also TCA-precipitated. Both samples were analyzed by immunoblotting in triplicate samples, and averages and SEMs were determined. Compared with *S. aureus* Newman (15.10% \pm 1.28% of released SpA), the *atl* (19.78% \pm 1.72; $P < 0.1$), *lytN* (16.69% \pm 1.44; $P < 0.5$), *sle1* (16.88% \pm 0.29; $P < 0.3$), or *atl/sle1/lytN* (17.80% \pm 0.49; $P < 0.5$) variants released similar amounts of protein A as WT staphylococci (Fig. 5A and B). In contrast, the *lytM* mutant strain released significantly less SpA into the culture medium than WT *S. aureus* (3.96% \pm 0.82; $P < 0.01$; Fig. 5A and B). These data indicate that the cross-wall murein hydrolases shape the cell wall anchor structure of SpA to ensure that released molecules lack the amino sugars of peptidoglycan. Nevertheless, cross-wall murein hydrolases are not responsible for the ultimate release of protein A from the bacterial envelope. This function can be assigned in part to LytM, which cuts the pentaglycyl cross-bridge of the protein A cell wall anchor structure.

Protein A Release During Staphylococcal Growth. We wondered whether SpA release occurs evenly during staphylococcal growth and followed stationary-phase *S. aureus* cells that were washed and diluted into fresh media in 30-min time intervals through logarithmic growth. Shortly following its dilution, i.e., during early

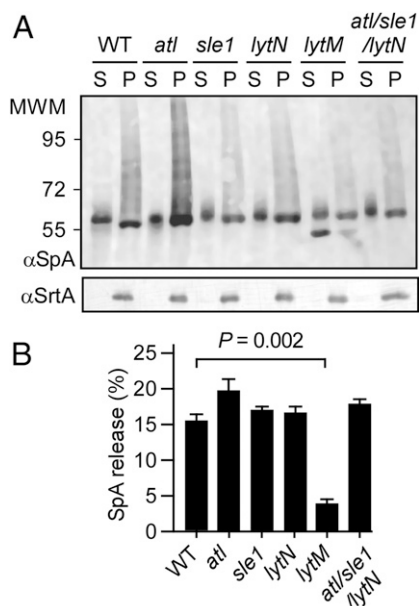


Fig. 5. LytM endopeptidase is required for protein A release from the staphylococcal envelope. (A) *S. aureus* WT and *atl*, *sle1*, *lytN*, *lytM*, or *atl/sle1/lytN* mutant cultures were centrifuged. Proteins in the culture media were separated with the supernatant (marked "S") from the bacterial pellet (marked "P"). After treatment of the staphylococcal cell wall envelope with lysostaphin, proteins in both fractions were analyzed by immunoblotting with polyclonal antibodies against protein A (α SpA) or sortase A (α SrtA). (B) Immune reactive signals from three different samples analyzed as shown in A were averaged, SEMs determined, and statistical significance analyzed with the two-tailed Student *t* test. Only the *lytM* mutant strain released less protein A than WT *S. aureus* ($P = 0.002$).

logarithmic growth, *S. aureus* released greater amounts of SpA per cfu than during later growth phases (Fig. 6). These results suggest that the release of SpA may be controlled by the growth phases of staphylococci, presumably with the intent of modifying host immune responses early during infection when numbers for this bacterial invader are small.

Discussion

Bacterial peptidoglycan is a potent activator of the mammalian immune system (40). Muropeptides released during bacterial growth are recognized by nucleotide-binding and oligomerization domain containing proteins 1 (NOD1) and 2 (NOD2), two members of the NLR family in the cytoplasm of mammalian cells that specialize in recognizing peptidoglycan fragments from Gram-negative (i.e., NOD1) or Gram-positive bacteria (i.e., NOD2) (41, 42). Activated by muropeptides with MurNAc, NOD1 and NOD2 initiate intracellular signaling pathways that induce NF- κ B and the expression of immune response genes encoding proinflammatory cytokines and antimicrobial peptides (40, 43). For example, neutrophil-derived IL-1 β production, a key defense against *S. aureus* (44), requires staphylococcal peptidoglycan-induced activation of NOD2 (45). The importance of this pathway is illustrated by defects in NOD2 signaling, which increases the disease susceptibility of mutant mice to *S. aureus* infection (46).

The NOD2 signaling pathway presents a challenge to the pathogenesis of *S. aureus* infections, in particular the suppression of B-cell responses (24, 25). How can pathogen-released protein A activate the clonal expansion of B cells and their subsequent apoptotic collapse without simultaneously triggering a systemic activation of the immune system? We show here that protein A is released from the envelope with linked peptidoglycan fragments, i.e., L-Ala-D-iGln-(Gly₅)L-Lys-D-Ala-Gly₄ and L-Ala-D-iGln-(Gly₅)

L-Lys-D-Ala-D-Ala. Of note these peptidoglycan fragments lack the amino sugars (MurNAc-GlcNAc) of peptidoglycan, which function as potent inducers of NOD2 (42). Pulse-labeling experiments indicate that protein A is initially tethered to the bacterial envelope, which occurs at the cross-wall between dividing daughter cells (14). Mutants lacking the cross-wall murein hydrolases—*Atl*, *Sle1*, or *LytN*—released SpA with altered peptidoglycan structures but did not affect the overall release of protein A. Thus, cross-wall murein hydrolases shape the cell wall anchor structure of protein A without affecting its release. The purpose of these activities appears to be the synthesis of SpA anchor structures that cannot stimulate host immune responses. When these tasks are completed, protein A remains tethered to the murein sacculi via its anchoring point, the peptidoglycan pentaglycine cross-bridge, which also connects neighboring wall peptides (7). *LytM* eventually cleaves these cross-bridges and releases protein A into the extracellular medium.

LytM is a secreted glycyl-glycine endopeptidase that cuts the cross-bridges of staphylococcal peptidoglycan without contributing to autolysis (39). Recent work revealed that *lytM* is a WalKR-regulated gene (47). In WalKR mutants, in which expression of the essential two-component regulator is limiting, *LytM* can suppress associated growth defects presumably by relaxing the cross-linking of peptidoglycan (48). Here, we suggest an important function for *LytM*: the release of biologically active sortase-anchored proteins from the bacterial envelope. As *lytM* mutants continue to release small amounts of protein A from the envelope, we surmise the existence of additional enzymes with glycyl-glycine endopeptidase activity that may impact the release of surface proteins.

Materials and Methods

Bacterial Strains and Growth Conditions. *S. aureus* strains were grown in TSB at 37 °C with agitation. *S. aureus* Newman WT (49) and variants with deletions in specific murein hydrolases *atl*, *sle1*, or *lytN* were generated by allelic replacement using the pKOR1 shuttle vector (50). Briefly, 1-kb flanking regions up- and downstream of *atl*, *sle1*, or *lytN* were amplified, ligated, and inserted into pKOR1 via lambda red recombination (50). Recombinant plasmids were electroporated into *S. aureus* Newman for integration into the chromosome and selection of deletion mutants. The *spa* mutant has been described earlier (51). To avoid contamination of protein A with the staphylococcal binder of Ig during affinity chromatography experiments (52), the *sbi::erm* allele described earlier (53) was transduced with ϕ 85 phage into *S. aureus* Newman as well as its *atl*, *sle1*, and *lytN* variants. Transductants *sbi::erm*, *atl/sbi::erm*, *sle1/sbi::erm*, and *lytN/sbi::erm* were confirmed by PCR and DNA

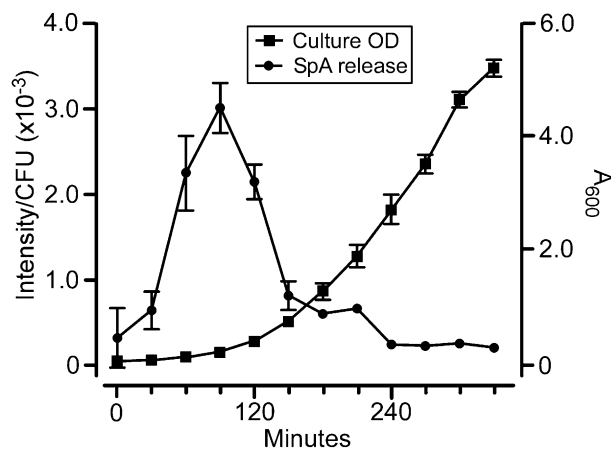


Fig. 6. SpA release during staphylococcal growth. *S. aureus* Newman (*sbi*) cells were washed and diluted into fresh TSB medium to A_{600} 0.05 and incubated with rotation at 37 °C. At 30-min intervals, the absorbance (600 nm) was measured and cfus were enumerated. SpA release was quantified by immunoblotting of culture supernatant samples and recorded as signal intensity divided by cfu ($\times 10^{-3}$). Experiments were performed in triplicate to calculate average values and SEM (error bars).

sequencing. The *bursa aurealis* transposon mutation Φ NE02987 (*lytM::erm*) was transduced with ϕ 85 into WT *S. aureus* Newman to generate the *lytM* mutant. Note that this mutant encodes the WT *sbj* gene.

Purification of SpA. *S. aureus* Newman and its variants were grown overnight in TSB and used to inoculate 8 L of fresh TSB with a 1:100 dilution inoculum. Cultures were grown for 4 h at 37 °C to absorbance at 600 nm (A_{600}) 0.2 and then centrifuged at $9,000 \times g$ for 20 min. The supernatant was decanted and cooled on ice to 4 °C, and 200 mL 1.5 M Tris-HCl (pH 8.8) added to adjust to pH 7.5. SpA molecules in the culture supernatant were purified by affinity chromatography. Briefly, a gravity-feed column with 4 mL bed volume IgG Sepharose6 Fast Flow was equilibrated with 14 mL elution buffer (0.1 M glycine-HCl, pH 3.0) followed by 30 mL Tris-saline-Tween 20 [TST; 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween 20]. A total of 8 L of culture supernatant was loaded onto the column at 4 °C over the course of 12 h. The column was washed with 100 mL TST and eluted with 14 mL elution buffer. The eluate was immediately neutralized with 0.35 mL 1.5 M Tris-HCl (pH 8.8). SpA molecules in 14 mL eluate were concentrated to a volume of 2 mL with a 10,000 molecular weight cut-off centrifugal filter unit (Millipore), dialyzed against PBS solution at 4 °C, and stored at -20 °C until further use. SpA purification was analyzed by Coomassie-stained SDS/PAGE, and protein concentration was determined with the BCA protein assay kit (Pierce).

MS. For structural analysis, 150 μ g purified SpA was dried, dissolved in 50 mL 5% (vol/vol) 2-mercaptoethanol, 0.2 M NH_4HCO_3 , and incubated overnight at room temperature. The protein sample was again dried and dissolved in 1 mL 70% formic acid. Approximately 1 mg cyanogen bromide was added to the solution. The reaction was incubated for 18 h in the dark at room temperature, and quenched by diluting the mixture 1:5 in water. The sample was then dried, dissolved in 30% acetonitrile, and desalted by reverse-phase HPLC via an acetonitrile/water gradient on C8 Hypersil solid-phase column. Eluate peaks were dried and dissolved in 5 to 20 μ L 30% acetonitrile. Protein samples were spotted on a ground platinum target plate (Bruker) and mixed 1:1 on the plate with α -cyano-4-hydroxycinnamic acid matrix (Sigma). Fractions were analyzed on a MALDI-TOF mass spectrometer (Autoflex Speed; Bruker) by using a reflectron-positive or linear-positive detection method. The detector was calibrated before each experiment with protein standards.

Measurement of SpA Release. *S. aureus* strains were inoculated from frozen stocks into TSB cultures and grown overnight. The culture was diluted to A_{600} 5.0, and 1 mL was centrifuged at $20,000 \times g$ for 1 min. The supernatant was discarded and staphylococci were washed two times in PBS solution. The bacterial suspension was inoculated into 100 mL TSB to yield A_{600} 0.05, and incubated at 37 °C. Before incubation and at timed intervals thereafter, 1.5 mL samples were removed from cultures and centrifuged at $20,000 \times g$ for 1 min. One milliliter of the supernatant was removed, placed in a fresh tube,

and stored on ice. The cell pellet was washed with 1 mL PBS solution and suspended in 1.5 mL TSM-L [50 mM Tris-HCl (pH 7.5), 0.5 M sucrose, 10 mM MgCl_2 , 5 $\mu\text{g}\cdot\text{mL}^{-1}$ lysostaphin (AMBI)] and placed at 37 °C for 15 min to digest the cell wall envelope with lysostaphin. A 1-mL aliquot of the staphylococcal protoplast suspension was then placed on ice.

Proteins in supernatant and protoplast samples were precipitated by adding 100% TCA/0.1% deoxycholic acid to a final concentration of 20%. Samples were vortexed and incubated on ice for 20 min. Precipitated proteins were sedimented by centrifugation at $22,000 \times g$ for 15 min. The supernatant was discarded, and protein pellets were washed twice with ice-cold acetone, air-dried, and solubilized in 25 μ L 0.5 M Tris-HCl (pH 8.0), 4% SDS at 4 °C overnight. An equal volume of sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue] was added, and protein samples were boiled for 10 min. Proteins were separated on 10% SDS-PAGE gel and electrotransferred to a PVDF membrane. The membrane was incubated in blocking buffer (TBS, 5% milk) for 1 h at room temperature. SpA_{KKAA} monoclonal antibody 3F6 was used at a 1:10,000 dilution in TBS-Tween 20. After 1 h, the membrane was washed three times and incubated in a 1:10,000 solution of goat anti-mouse 680 antibody (Licor). After 1 h, the membrane was washed three times, and fluorescence was measured at 700 nm on an infrared scanner (Licor Odyssey). Signal integration was performed by Licor software. To ensure consistency between blots, two standard dilutions of purified SpA were included in every blot as a fluorescence control.

SpA Release During Staphylococcal Growth. Overnight cultures of *S. aureus* Newman *sbj* were centrifuged, and bacterial cells were washed and diluted into fresh TSB medium to A_{600} 0.05 and incubated with rotation at 37 °C. At 30-min intervals, the absorbance at 600 nm was measured and culture aliquots were plated to enumerate cfus. Additionally, 1.5-mL culture aliquots were removed to quantify protein A release. Following centrifugation of culture samples, 1 mL supernatant was withdrawn and proteins precipitated with TCA. The precipitate was solubilized in sample buffer and subjected to immunoblotting to quantify the abundance of SpA. The rate of SpA release was calculated by dividing the intensity of SpA-immunoreactive signals with the cfu measurements for each time interval. Experiments were performed in triplicate to calculate average values and SEM.

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