THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 18, Issue of May 3, pp. 16241–16248, 2002
Printed in U.S.A.

Anchoring of Surface Proteins to the Cell Wall of *Staphylococcus aureus*

III. LIPID II IS AN *IN VIVO* PEPTIDOGLYCAN SUBSTRATE FOR SORTASE-CATALYZED SURFACE PROTEIN ANCHORING*

Received for publication, September 24, 2001, and in revised form, February 18, 2002
Published, JBC Papers in Press, February 20, 2002, DOI 10.1074/jbc.M109194200

Adrienne M. Perry, Hung Ton-That, Sarkis K. Mazmanian, and Olaf Schneewind‡

From the Committee on Microbiology, University of Chicago, Chicago, Illinois 60637

Surface proteins of *Staphylococcus aureus* are anchored to the cell wall peptidoglycan by a mechanism requiring a C-terminal sorting signal with an LP*X*TG motif. Surface proteins are first synthesized in the bacterial cytoplasm and then transported across the cytoplasmic membrane. Cleavage of the N-terminal signal peptide of the cytoplasmic surface protein P1 precursor generates the extracellular P2 species, which is the substrate for the cell wall anchoring reaction. Sortase, a membrane-anchored transpeptidase, cleaves P2 between the threonine (T) and the glycine (G) of the LP*X*TG motif and catalyzes the formation of an amide bond between the carboxyl group of threonine and the amino group of cell wall cross-bridges. We have used metabolic labeling of staphylococcal cultures with [^{32}P]phosphoric acid to reveal a P3 intermediate. The ^{32}P-label of immunoprecipitated surface protein is removed by treatment with lysostaphin, a glycyl-glycine endopeptidase that separates the cell wall anchor structure. Furthermore, the appearance of P3 is prevented in the absence of sortase or by the inhibition of cell wall synthesis. ^{32}P-Labeled cell wall anchor species bind to nisin, an antibiotic that is known to form a complex with lipid II. Thus, it appears that the P3 intermediate represents surface protein linked to the lipid II peptidoglycan precursor. The data support a model whereby lipid II-linked polypeptides are incorporated into the growing peptidoglycan via the transpeptidation and transglycosylation reactions of cell wall synthesis, generating mature cell wall-linked surface protein.

To mount a successful infection, Gram-positive pathogens display proteins on the bacterial surface that adhere to specific receptors on host tissues or provide for microbial escape from the host's immune response (1). Protein display on the bacterial surface involves the covalent linkage of polypeptides to the cell wall envelope (2). As reported for protein A of *Staphylococcus aureus*, surface proteins are synthesized as P1 precursor molecules in the bacterial cytoplasm, bearing an N-terminal signal peptide and a C-terminal sorting signal (3). The 35-residue sorting signal is composed of a LP*X*TG motif, a hydrophobic domain, and a tail of positively charged residues (4). After translocation across the cytoplasmic membrane, the N-terminal signal peptide is removed by signal peptidase, thereby generating the P2 precursor (4). The C-terminal sorting signal retains the P2 precursor species within the secretory pathway and permits substrate recognition at the LP*X*TG motif (4, 5). Sortase, a membrane-anchored transpeptidase, cleaves surface proteins between the threonine (T) and the glycine (G) of the LP*X*TG motif (6, 7). Cleaved polypeptides are initially tethered as thioester-linked intermediates to the active site sulfhydryl residue of sortase enzymes (8). Nucleophilic attack of the amino group of pentaglycine cross-bridges within the staphylococcal peptidoglycan resolves this acyl-enzyme intermediate (8), resulting in the formation of an amide bond that tethers the C terminus of surface protein to the cell wall peptidoglycan (9–13).

The peptidoglycan of *S. aureus* is synthesized in three cellular compartments, the cytoplasm, the membrane and the cell wall envelope (14). The soluble cytoplasmic peptidoglycan precursor UDP-MurNAc-L-Ala-D-iGln-L-Lys-D-Ala-D-Ala[1] (Park's nucleotide) is linked to the membrane lipid undecaprenolphosphate, generating lipid I (undecaprenolpyrophosphate-MurNAc-L-Ala-D-iGln-(NH_2)-L-Lys-D-Ala-D-Ala) (15–17). Lipid I is modified by the addition of GlcNAc and pentaglycine to yield lipid II (undecaprenolpyrophosphate-MurNAc(-L-Ala-D-iGln-(NH_2-Gly_5)-L-Lys-D-Ala-D-Ala)-(β1–4)-GlcNAc) (18, 19). Lipid II is translocated across the cytoplasmic membrane and functions as a substrate for two cell wall biosynthetic reactions that require mono- or bifunctional transglycosylases and transpeptidases (20). In the transglycosylation reaction, lipid II is polymerized to generate linear peptidoglycan strands with the repeating disaccharide $(MurNAc\text{-}GlcNAc)_n$. This reaction is fueled by the hydrolysis of lipid II and by further hydrolysis of the undecaprenolpyrophosphate product, which is translocated across the plasma membrane into the cytoplasm (21). Linear peptidoglycan strands are cross-linked by transpeptidases that cleave murein pentapeptides (L-Ala-D-iGln-(NH_2-Gly_5)-L-Lys-D-Ala-D-Ala) and synthesize an amide bond between the carboxyl group of L-Ala-D-iGln-(NH_2-Gly_5)-L-Lys-D-Ala-COOH and the amino group of pentaglycine cross-bridges (NH_2-Gly_5) within neighboring peptidoglycan strands (22, 23). Together the transglycosylation and transpeptidation reactions generate the three-dimensional network of mature peptidoglycan, which in

* This work was supported by United States Public Health Service Grant AI38897. This is Paper III in the series *Anchoring of Surface Proteins to the Cell Wall of Staphylococcus aureus*. Refs. 51 and 52 are Papers I and II, respectively. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Committee on Microbiology, The University of Chicago, 920 East 58th St., Chicago, IL 60637. Tel.: 773-834-9060; Fax: 773-834-8150; E-mail: oschnee@delphi.bsd.uchicago.edu.

[1] The abbreviations used are: MurNAc, *N*-acetylmuramic acid; Cws, cell wall sorting signal; GlcNAc, *N*-acetylglucosamine; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate; Seb, staphylococcal enterotoxin B; TSB, tryptic soy broth; iGln, D-isoglutamine; RIPA, radioimmune precipitation buffer; pHTT4, [^{32}P]phosphoric acid-labeled *S. aureus* RN4220.

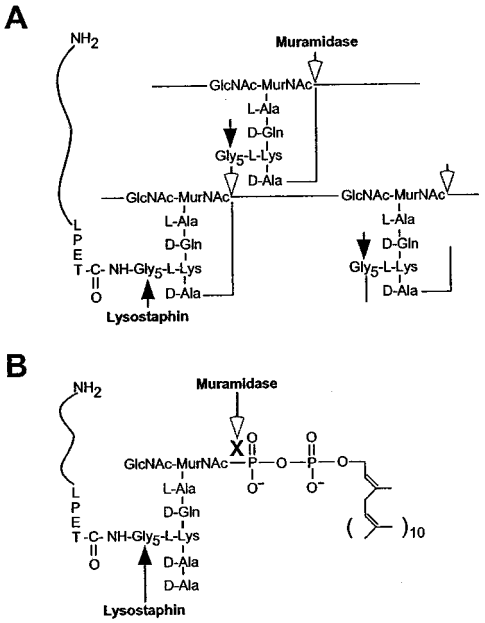

FIG. 1. **The structure of *S. aureus* cell wall-anchored surface protein and of the P3 intermediate.** *A*, structure of surface protein linked by an amide bond between the C-terminal threonine of the LPXTG motif and the amino group of the pentaglycine cross-bridge. Also shown is the structure of cross-linked peptidoglycan and the cleavage sites for lysostaphin (glycyl-glycine endopeptidase) and muramidase (MurNAc-(β1–4)-GlcNAc). *B*, presumed structure of surface protein linked to lipid II (P3 precursor). Lysostaphin is expected to cleave surface protein off lipid II, however, muramidase cannot cleave the P3 precursor structure.

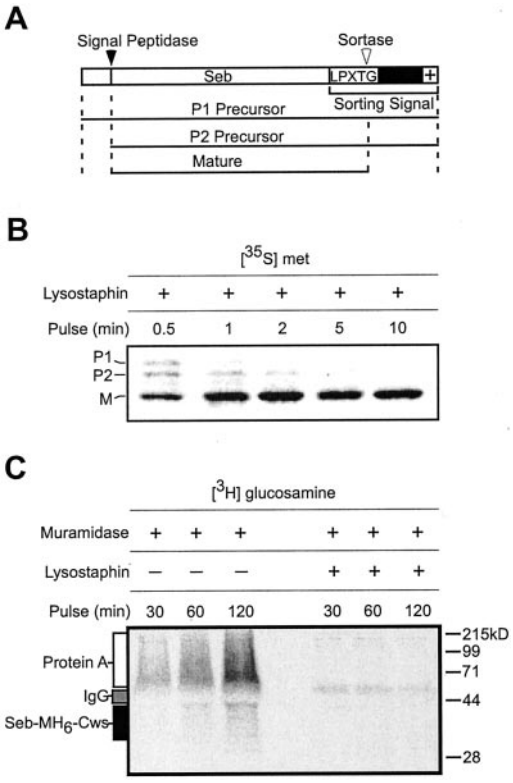

FIG. 2. **Incorporation of [^{35}S]methionine and [^{3}H]*N*-acetylglucosamine into *S. aureus* surface protein.** *A*, primary structure of the surface protein precursor Seb-MH_6-Cws, a fusion between enterotoxin B (*Seb*) and C-terminal cell wall sorting signal (*Cws*) of protein A. P1 precursor is directed across the cytoplasmic membrane by an N-terminal leader peptide and is then cleaved by signal peptidase to generate P2. P2 bears a C-terminal sorting signal that includes an LPXTG motif, a hydrophobic domain (*black bar*) and positively charged tail (*boxed* +). The sorting signal of P2 is cleaved at the LPXTG motif and the mature protein (*M*) is linked to the cell wall. *B*, *S. aureus* RN4220 (pHTT4) were pulse-labeled with [^{35}S]methionine. At timed intervals, *i.e.* 0.5, 1, 2, 5, and 10 min after the addition of an excess unlabeled methionine, cells and proteins were precipitated with trichloroacetic acid and the peptidoglycan was digested with lysostaphin. Following immunoprecipitation, surface protein was separated on SDS-PAGE and analyzed by phosphorimaging. *C*, *S. aureus* RN4220 (pHTT4) (protein A and Seb-MH_6-Cws) were subjected to labeling with [^{3}H]GlcNAc followed by mutanolysin digestion of the cell wall, immunoprecipitation, SDS-PAGE, and PhosphorImager analysis. Some immunoprecipitated surface protein was digested with lysostaphin prior to SDS-PAGE.

staphylococci contains less than 1% of free (non-cross-linked) amino groups (NH_2-Gly_5) and glycan chains that are 12–60 sugar residues in length (24, 25).

Treatment of staphylococci with the strong nucleophile hydroxylamine releases surface protein acyl-intermediate from sortase into the extracellular medium (8). The released surface proteins bear a C-terminal threonine hydroxamate. These results suggest that the active site of sortase enzymes in staphylococci may be generally occupied with cleaved polypeptides. Thus, the rate-limiting step in surface protein anchoring appears to be the nucleophilic attack of the peptidoglycan substrate that regenerates the active site sulfhydryl of sortase (26). What is the peptidoglycan substrate that performs the nucleophilic attack? Previous work addressed this question using two experimental approaches. By following [^{35}S]methionine-labeled polypeptides over time, it was determined that surface protein cleavage at the LPXTG motif occurred both in intact bacteria and in staphylococcal protoplasts, cells in which the peptidoglycan envelope had been removed by enzymatic digestion (27). The second approach tested inhibitors of cell wall synthesis for their effect on surface protein anchoring. Vancomycin binds to the D-Ala-D-Ala moiety of lipid II (28, 29) and prevents both transglycosylase and transpeptidase reactions (30). In contrast, moenomycin is an inhibitor of transglycosylation alone (31). Addition of vancomycin caused peptidoglycan synthesis inhibition and a steady accumulation of P2 precursor, indicating that this compound causes a reduction of surface protein anchoring (27). A similar effect was observed when moenomycin was added to staphylococcal cultures (27). Together these results suggest that sortase utilizes a peptidoglycan precursor, but not mature assembled cell wall, as a substrate for surface protein anchoring.

In this report we have labeled *S. aureus* cells with [^{32}P]phosphoric acid and revealed the P3 intermediate of surface protein anchoring. The P3 intermediate likely represents surface protein linked to lipid II and functions as a substrate for the transglycosylation and transpeptidation reactions that incorporate surface protein into the peptidoglycan envelope.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—S. aureus RN4220 (sortase wild-type) and its isogenic variant SKM1 (*srtA:ermC*) have been previously described (32, 33). Plasmid pHTT4 encodes Seb-MH_6-Cws, an engineered surface protein that has been characterized extensively in structural analysis of cell wall anchoring (10). All chemicals were purchased from Sigma Chemical Co. unless indicated otherwise.

Minimal medium lacking phosphate (MM-PO_4) was generated by the assembly of the following components: amino acid solution I (10×: threonine, serine, alanine, proline, valine, leucine, isoleucine, phenylalanine, asparagine, glutamine, lysine, arginine, histidine, and glycine at 1.2% each in water), amino acid solution II (20×: aspartic acid, glutamic acid, and tyrosine at 1% each in 0.4 M sodium hydroxide), amino acid solution III (100×: tryptophan at 1% in 0.2 M HCl), salt-PO_4 solution (4×: 4 g of ammonium sulfate, 2 g of sodium citrate × $2H_2O$, 3.125 g of Tris-HCl, 400 mg of magnesium sulfate × $7H_2O$, 40 mg of ferrous sulfate × $7H_2O$, water to 1 liter and pH 7.0), vitamin solution (500×: 500 mg of niacin, 25 mg of thiamine-HCl, and water to 0.1 liter), glucose solution (50×: 20% dextrose in water). To assemble 50 ml of

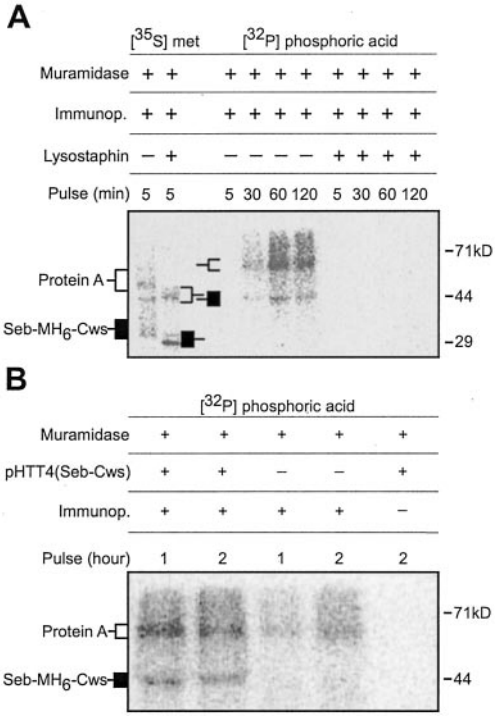

FIG. 3. **Labeling *S. aureus* surface protein with [^{32}P]phosphoric acid.** *A*, *S. aureus* RN4220 (pHTT4), expressing protein A and Seb-MH_6-Cws, was labeled with either [^{35}S]methionine or [^{32}P]phosphoric acid. Cells and proteins were precipitated with trichloroacetic acid, and the peptidoglycan was digested with muramidase. Following immunoprecipitation, surface proteins were either left untreated or cut with lysostaphin and separated on SDS-PAGE and analyzed by phosphorimaging. *B*, *S. aureus* RN4220 (pHTT4) (protein A and Seb-MH_6-Cws) and *S. aureus* RN4220 (protein A but no Seb-MH_6-Cws) were subjected to labeling with [^{32}P]phosphoric acid followed by immunoprecipitation, SDS-PAGE, and PhosphorImager analysis. As a control for antibody specificity, samples were subjected to the same protocol without added antiserum.

MM-PO_4, 28.4 ml of deionized water, 12.5 ml of 4× salt-PO_4, 5 ml of amino acid solution I, 2.5 ml of amino acid solution II, 0.5 ml of amino acid solution III, 0.1 ml of vitamin solution, and 1 ml of dextrose solution were mixed, warmed to 37 °C, and supplemented with 10 μg/ml chloramphenicol. Minimal medium (MM) with phosphate was generated as described for MM-PO_4 and substituting the 4× salt-PO_4 with 4× salt solution (4 g of ammonium sulfate, 2 g of sodium citrate × $2H_2O$, 8 g of potassium dihydrogen phosphate, 8 g of disodium hydrogen phosphate, 400 mg of magnesium sulfate × $7H_2O$, 40 mg of ferrous sulfate × $7H_2O$, deionized water to 1 liter and pH 7.5).

Labeling with [^{32}P]Phosphoric Acid—S. aureus strains RN4220 (pHTT4) and SKM1 (pHTT4) were grown overnight in TSB supplemented with 10 μg/ml chloramphenicol. Cultures (1 ml) were diluted into 25 ml of fresh medium and grown with vigorous shaking for 3 h at 37 °C. Cultures were then centrifuged at 8000 × *g* for 7 min. The bacterial sediment was washed twice in an equal volume of MM-PO_4 and suspended in a volume of MM-PO_4 to yield an A_{600} 1.2 and used immediately for labeling experiments. Four milliliters of cells were labeled by adding 200 μCi of [^{32}P]phosphoric acid, mixed, and incubated in a 37 °C water bath. At timed intervals (5, 30, 60, and 120 min), 1 ml of cells was removed, transferred into an Eppendorf tube, and all further incorporation of [^{32}P]phosphoric acid into bacterial cell structures was quenched by the addition of 7.5% trichloroacetic acid and incubation on ice for 30 min. Total cells and precipitated molecules were collected by centrifugation at 16,000 × *g* for 10 min, washed in ice-cold acetone, precipitated by centrifugation at 16,000 × *g* for 10 min, and dried. Samples were suspended in 1 ml of 0.5 M Tris-HCl, pH 6.3, and peptidoglycan was digested by adding 150 μg of mutanolysin and incubation for 4 h at 37 °C with intermittent mixing of samples. Mutanolysin digests were precipitated by the addition of 7.5% trichloroacetic acid and incubation on ice for 30 min. The precipitate was collected by centrifugation at 16,000 × *g* for 10 min, washed in ice-cold acetone, precipitated by centrifugation at 16,000 × *g* for 10 min and dried. Samples were solubilized by boiling in 50 μl of 0.5 M Tris-HCl, 4% SDS, pH 8.0. 40-μl samples were transferred to 1 ml of RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, pH 8.0) containing 1 μl of rabbit α-Seb antibodies. Antigen-antibody complexes were captured on 50 μl of pre-swollen protein A CL 4B-Sepharose, washed five times with RIPA buffer and solubilized by boiling in sample buffer. Immunoprecipitates were separated on 14% SDS-PAGE, dried, and analyzed on PhosphorImager.

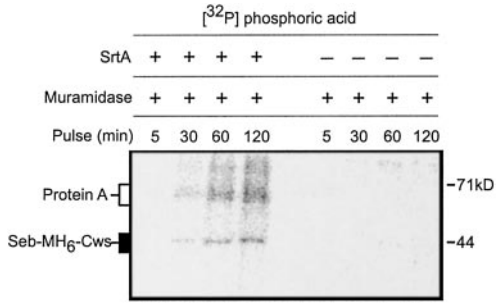

FIG. 4. **Sortase is required for the biosynthesis of ^{32}P-labeled P3 intermediate.** *A*, *S. aureus* RN4220 (pHTT4) and *S. aureus* SKM1 (*srtA:ermC*) (pHTT4) were subjected to labeling with [^{32}P]phosphoric acid followed by immunoprecipitation, SDS-PAGE, and PhosphorImager analysis. Incorporation of ^{32}P into *S. aureus* RN4220 (pHTT4) and *S. aureus* SKM1 (*srtA:ermC*) (pHTT4) was determined by subjecting trichloroacetic acid-precipitated aliquots to liquid scintillation counting (see Table I for quantification).

Labeling with [^{35}S]Methionine—S. aureus strains RN4220 (pHTT4) and SKM1 (pHTT4) were grown overnight in TSB supplemented with 10 μg/ml chloramphenicol. Cultures (1 ml) were diluted into 25 ml of fresh medium and grown with vigorous shaking for 3 h at 37 °C. Cultures were then centrifuged at 8000 × *g* for 15 min. The bacterial sediment was washed twice in an equal volume of MM and suspended in MM at A_{600} 1.2 and used immediately for pulse labeling. 1 ml of cells was labeled by adding 100 μCi of [^{35}S]methionine, mixed, and incubated in a 37 °C water bath. After 2 min of labeling, 50 μl of chase solution was added (100 mg/ml casamino acids, 10 mg/ml each of methionine and cysteine) and at timed intervals (0, 1, 5, and 20 min after the chase), 250 μl of cells was removed, transferred into an Eppendorf tube, and all further processing of surface proteins was quenched by the addition of 7.5% trichloroacetic acid and incubation on ice for 30 min. Total cells and precipitated molecules were collected by centrifugation at 16,000 × *g* for 10 min, washed in ice-cold acetone, precipitated by centrifugation at 16,000 × *g* for 10 min, and dried. Samples were suspended in 1 ml of 0.5 M Tris-HCl, pH 6.3, and peptidoglycan digested by adding 150 μg of mutanolysin and incubation for 4 h at 37 °C with intermittent mixing of samples. Mutanolysin digests were precipitated by the addition of 7.5% trichloroacetic acid and incubation on ice for 30 min. (Some samples were directly digested with lysostaphin, *i.e.* 100 μg of recombinant lysostaphin and incubation for 1 h at 37 °C with intermittent mixing.) The precipitate was collected by centrifugation at 16,000 × *g* for 10 min, washed in ice-cold acetone, precipitated by centrifugation at 16,000 × *g* for 10 min, and dried. Samples were solubilized by boiling in 50 μl of 0.5 M Tris-HCl, 4% SDS, pH 8.0. 40-μl samples were transferred to 1 ml of RIPA buffer containing 1 μl of rabbit α-Seb antibodies. Antigen-antibody complexes were captured on 50 μl of pre-swollen protein A CL 4B-Sepharose, washed five times with RIPA buffer, and solubilized by boiling in sample buffer. Immunoprecipitates were separated on 14% SDS-PAGE, dried, and analyzed on PhosphorImager.

Labeling with [^{3}H]N-Acetylglucosamine—S. aureus strains RN4220 (pHTT4) was grown overnight in TSB supplemented with 10 μg/ml chloramphenicol. Cultures (1 ml) were diluted into 25 ml of fresh medium and grown with vigorous shaking for 3 h at 37 °C. Cultures were then centrifuged at 8000 × *g* for 7 min. The bacterial sediment was washed twice in an equal volume of MM and suspended in a volume of MM to yield an A_{600} 1.2 and used immediately for labeling experiments. Three milliliters of cells were labeled by adding 150 μCi of [^{3}H]GlcNAc, mixed, and incubated in a 37 °C water bath. At timed intervals (30, 60, and 120 min), 1 ml of cells was removed, transferred into an Eppendorf tube, and all further incorporation of [^{3}H]GlcNAc into bacterial cell structures was quenched by the addition of 7.5% trichloroacetic acid and incubation on ice for 30 min. Total cells and precipitated molecules were collected by centrifugation at 16,000 × *g* for 10 min, washed in ice-cold acetone, precipitated by centrifugation at 16,000 × *g* for 10 min, and dried. Samples were suspended in 1 ml of 0.5 M Tris-HCl, pH 6.3, and peptidoglycan was digested by adding 150 μg of mutanolysin and

TABLE I
Sortase is required for the synthesis of surface protein P3 precursor
Data were obtained and quantified from Fig. 4.

Surface protein	^{32}P-Surface protein P3/total cellular ^{32}P concentration			
	5 min[a]	30 min	60 min	120 min
Wild-type[b]				
Protein A	5	43	39	27
Seb-MH$_6$-Cws	0	14	12	9
srtA$^-$[c]				
Protein A	0	2	2	2
Seb-MH$_6$-Cws	0	1	1	1

[a] Time after the addition of [^{32}P]phosphoric acid to *S. aureus* RN4220 (pHTT4) culture at which labeling was quenched with ice-cold trichloroacetic acid.
[b] *S. aureus* RN4220.
[c] *S. aureus* SKM1 (*srtA*$^-$).

incubation for 4 h at 37 °C with intermittent mixing of samples. Mutanolysin digests were precipitated by the addition of 7.5% trichloroacetic acid and incubation on ice for 30 min. The precipitate was collected by centrifugation at 16,000 × *g* for 10 min, washed in ice-cold acetone, precipitated by centrifugation at 16,000 × *g* for 10 min, and dried. Samples were solubilized by boiling in 50 μl of 0.5 M Tris-HCl, 4% SDS, pH 8.0. 40-μl samples were transferred to 1 ml of RIPA buffer containing 1 μl of rabbit α-Seb antibodies. Antigen-antibody complexes were captured on 50 μl of pre-swollen protein A CL 4B-Sepharose, washed five times with RIPA buffer, and solubilized by boiling in sample buffer. Immunoprecipitates were separated on 14% SDS-PAGE, transferred to polyvinylidene difluoride, dried, and analyzed via tritium screen on a PhosphorImager instrument.

Lysostaphin Digestion of Immunoprecipitated Surface Protein—After washing immunoprecipitated samples with RIPA buffer, two washes with 0.1 M Tris-HCl, pH 7.0, were added, and antigen antibody complexes bound to protein A-Sepharose beads were suspended in 1 ml of 0.1 M Tris-HCl, pH 7.0, with 200 μg of lysostaphin and incubated for 1 h. After another round of washing in RIPA buffer, the antigen/antibody complexes were disrupted by boiling in sample buffer and separated on 14% SDS-PAGE.

Inhibition of Surface Protein Anchoring in S. aureus Cultures—*S. aureus* RN4220 (pHTT4) was grown overnight in TSB supplemented with 10 μg/ml chloramphenicol. Cultures (1 ml) were diluted into 25 ml of fresh medium supplemented and grown with vigorous shaking for 30 min at 37 °C. At this time, 10 μg/ml penicillin G, 10 μg/ml vancomycin, 10 μg/ml moenomycin, 20 μg/ml bacitracin, or 15 μg/ml tunicamycin were added, and the cultures were grown with vigorous shaking for an additional 150 min at 37 °C. The A_{600} was recorded at timed intervals, and washed cells were suspended in small volumes to achieve labeling of 1.2 A_{600} units of cells with [^{32}P]phosphoric acid.

Measuring the Incorporation of Radiolabel by S. aureus Cultures—To determine the amount of incorporated radiolabel, trichloroacetic acid precipitated, and acetone washed samples (after mutanolysin or lysostaphin digestion) were suspended in 50 μl of 0.5 M Tris-HCl, 4% SDS, pH 8.0, and 2-μl aliquots were subjected to scintillation counting.

Immunoblotting—Mutanolysin-digested and trichloroacetic acid precipitated cell extracts were suspended by boiling in 50 μl of 0.5 M Tris-HCl, 4% SDS, pH 8.0. An equal amount of sample buffer was added, and samples were heated to 95 °C. for 10 min, 10-μl aliquots were separated on SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Millipore). The membrane filter was blocked in 20 ml of TBS-M (20 mM Tris-HCl, 20 mM NaCl, 0.1% Tween 20, pH 7.5, and 5% dried skim milk). The filter was washed in TBS-T (TBS-M without milk) and incubated for 1 h with a 1:20,000 dilution of monoclonal antibody SPA-27 in 20 ml of TBS-M. The filter was again washed three times in TBS-T and incubated for 45 min with a 1:5000 diluted secondary antibody (anti-mouse conjugated to horseradish peroxidase). Immunoreactive signals were developed with a chemiluminescent substrate using an AlphaImager.

Thin-layer Chromatography—[^{32}P]Phosphoric acid-labeled surface protein was immunoprecipitated from mutanolysin-digested staphylococci. After five washes with RIPA buffer, the charged protein A CL-4B-Sepharose beads were pooled, washed with 0.1 mM Tris-HCl (pH 7.0), and dispensed into aliquots. After removal of the buffer, samples were incubated for 1 h at 37 °C by: (i) mock treatment with 20 μl of 25 mM Tris-HCl, pH 7.0, or (ii) treatment with 8 μg of lysostaphin in 25 mM Tris-HCl, pH 7.0. To measure the formation of a complex between

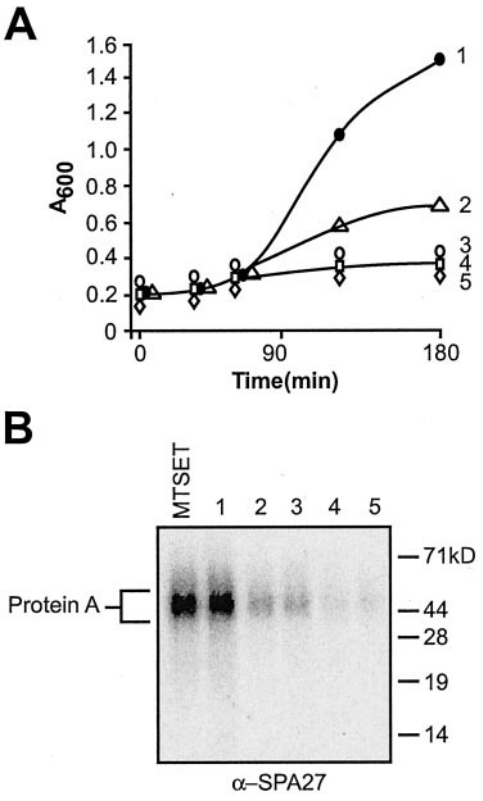

FIG. 5. **Antibiotic inhibition of peptidoglycan synthesis and surface protein display.** *A*, *S. aureus* RN4220 (pHTT4) cells were diluted into fresh TSB medium containing either 10 μg/ml chloramphenicol and no antibiotic (*1*), 20 μg/ml bacitracin (*2*), 10 μg/ml penicillin (*3*), 10 μg/ml vancomycin (*4*), or 10 μg/ml moenomycin (*5*). At timed intervals, the cell density of culture aliquots was determined as the A_{600}. *B*, culture aliquots obtained after 150 min of antibiotic inhibition were precipitated with trichloroacetic acid, and the peptidoglycan was digested with muramidase. Aliquots were separated on SDS-PAGE and analyzed by immunoblotting with protein A-specific monoclonal antibody (α-SPA27). One culture aliquot of staphylococci that were grown in the absence antibiotics was treated for 5 min with MTSET, a known inhibitor of sortase.

^{32}P-labeled cell wall anchor structures and nisin, 20 μl of lysostaphin-digested sample was mixed with 20 μl of mock mix (644.8 μl of water, 100 μl of 1 M Tris-HCl, pH 8.8, 116 μl of 1 M $MgCl_2$, 23.2 μl of 1 M NH_4Cl, 116 μl of 0.1 M SDS) or 20 μl of nisin mix (624.8 μl of water, 100 μl of 1 M Tris-HCl, pH 8.8, 116 μl of 1 M $MgCl_2$, 23.2 μl of 1 M NH_4Cl, 116 μl of 0.1 M SDS, 20 μl of nisin, 80 mg/ml water). Reactions were incubated at 25 °C for 1.5 h. For TLC analysis, 2-μl aliquots were spotted onto K6 silica plates and separated 7 cm using one of two solvents: solvent A is isobutyric acid and 1 N NH_4OH (5:3, v/v); solvent B is *n*-butanol, acetic acid, water, pyridine (15:3:12:10, v/v). Silica gels were dried and placed on PhosphorImager screens for 3 days.

RESULTS

[^{32}P]Phosphoric Acid-labeled Surface Protein—An earlier model for the cell wall-anchoring reaction predicted the existence of the P3 intermediate, a compound in which surface protein is amide-linked to the cross-bridge of lipid II (10) (Fig. 1). However, pulse-labeling experiments with [^{35}S]methionine revealed processing of P1 and P2 precursors to mature anchored surface protein but failed to detect the P3 intermediate (Fig. 2, *A* and *B*). During the course of these experiments the cell wall envelope of staphylococci was treated with lysostaphin, a glycyl-glycine endopeptidase that cleaves *S. aureus* cross-bridges (34), thereby solubilizing P1, P2, and mature surface protein. Close examination of the structure of the presumed P3 intermediate and of mature surface protein revealed that both compounds contain pentaglycine cross-bridges as anchoring points for surface proteins (Fig. 1). Thus, after cleavage

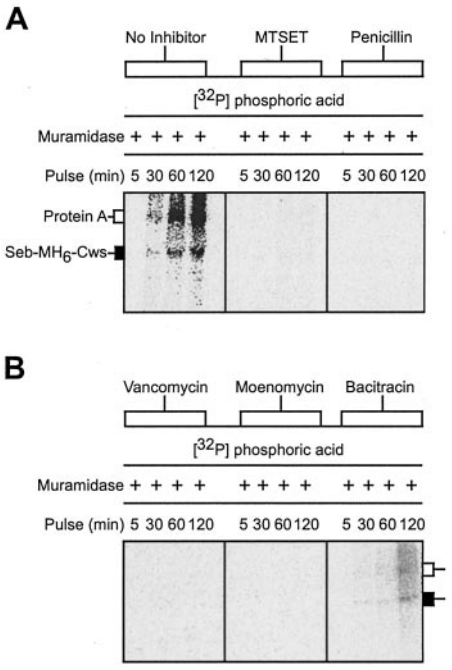

FIG. 6. **Antibiotic inhibition of the biosynthesis of ^{32}P-labeled P3 intermediate.** *A*, *S. aureus* RN4220 (pHTT4) cells were diluted into fresh TSB medium containing either 10 μg/ml chloramphenicol and no antibiotic (no inhibitor), penicillin, vancomycin, moenomycin, or bacitracin. 4 ml of cells was labeled with [^{32}P]phosphoric acid for 5, 30, 60, and 120 min. At timed intervals, 1-ml samples were precipitated with trichloroacetic acid, digested with muramidase, and analyzed by immunoprecipitation, SDS-PAGE, and phosphorimaging. The sortase inhibitor MTSET was added shortly after labeling with [^{32}P]phosphoric acid.

TABLE II
Bacitracin inhibits the synthesis of surface protein P3 precursor
Data were obtained and quantified from Fig. 6.

Surface protein	^{32}P-Surface protein P3/total cellular ^{32}P concentration			
	5 min[a]	30 min	60 min	120 min
No inhibitor				
Protein A	7	10	28	19
Seb-MH$_6$-Cws	1	6	11	9
Bacitracin				
Protein A	0	2	1.5	11
Seb-MH$_6$-Cws	0	1	1	7

[a] Time after the addition of [^{32}P]phosphoric acid to *S. aureus* RN4220 (pHTT4) culture at which labeling was quenched with ice-cold trichloroacetic acid.

with lysostaphin, it is impossible to distinguish P3 precursor and mature surface protein because both species display the same compound structure and mass. Mutanolysin, an *N*-acetylmuramidase that cuts the β1–4 glycosidic bond between MurNAc-GlcNAc, cleaves the glycan strands of mature peptidoglycan but does not cut lipid II or surface protein linked to lipid II (35). Mutanolysin-released surface protein migrates as a large spectrum of fragments on SDS-PAGE, a phenomenon that precludes the identification of [^{35}S]methionine-labeled P3 precursors with discrete mass (4) (Fig. 3).

Mature surface protein is linked to peptidoglycan, a polymer that contains the repeating disaccharide MurNAc-GlcNAc. Staphylococcal cultures were labeled with [^{3}H]GlcNAc. Bacteria were isolated and their cell walls digested with mutanolysin. Surface protein was immunoprecipitated and analyzed by SDS-PAGE and PhosphorImager after amplification of the tritium signal (Fig. 2*C*). A spectrum of [^{3}H]GlcNAc-labeled protein A and Seb-MH$_6$-Cws molecules with linked cell wall fragments

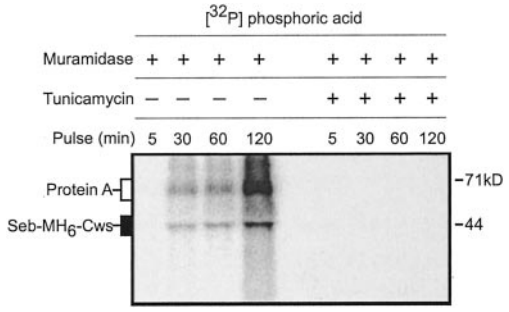

FIG. 7. **Tunicamycin inhibits the formation of ^{32}P-labeled P3 intermediate.** *A*, *S. aureus* RN4220 (pHTT4) cells were diluted into fresh TSB medium containing either 10 μg/ml chloramphenicol and no antibiotic (–) or tunicamycin (+). 4 of cells was labeled with [^{32}P]phosphoric acid for 5, 30, 60, and 120 min. At timed intervals, 1-ml samples were precipitated with trichloroacetic acid, digested with muramidase, and analyzed by immunoprecipitation, SDS-PAGE, and phosphorimaging.

was detected. Lysostaphin digestion of the mutanolysin-released and immunoprecipitated surface protein removed all [^{3}H]GlcNAc, indicating that polypeptides were indeed linked to peptidoglycan. (The *single radioactive band* in Fig. 2*C* is caused by the compression of IgG heavy chains and represents an artifact of immunoprecipitation.) As the products of the sorting reaction, *i.e.* cell wall-anchored surface protein, are far more abundant than the P3 precursor species, it appears that alternative labeling techniques are needed to demonstrate the formation of lipid II-linked surface protein.

Previous work achieved labeling of *S. aureus* undecaprenol-phosphate, lipid I, and lipid II with [^{32}P]phosphoric acid (36, 37) (see Fig. 1. for a *structure* of lipid II). We reasoned that [^{32}P]phosphoric acid labeling of staphylococci may allow incorporation of [^{32}P] into surface protein P3 precursor species. Because lipid II is the only extracellular peptidoglycan precursor that is known to harbor phosphate (38), isolation of ^{32}P-labeled surface protein would provide strong evidence for the linkage of polypeptides to lipid II. To test this prediction, *S. aureus* RN4220 (pHTT4), a strain expressing the immunoglobulin binding protein A (39) and Seb-MH$_6$-Cws (an engineered surface protein encoded on pHTT4), was grown in TSB. The cells were collected by centrifugation, washed, and suspended in minimal medium lacking phosphate. After adding [^{32}P]phosphoric acid for various amounts of time, labeling was quenched by precipitating all proteins with ice-cold trichloroacetic acid. The cell walls of staphylococci were digested with mutanolysin. Protein A and Seb-MH$_6$-Cws were immunoprecipitated with specific antibody, separated on SDS-PAGE, and detected by PhosphorImager. This approach revealed two ^{32}P-labeled surface protein species, migrating on SDS-PAGE with a mass of 44 and 54 kDa (Fig. 3*A*). Labeling with [^{35}S]methionine followed by muramidase digestion and immunoprecipitation identified cell wall-anchored protein A and Seb-MH$_6$-Cws, each of which migrated as a spectrum of fragments with linked peptidoglycan (4). We sought to obtain definitive proof for the notion that the faster migrating species represented [^{32}P]Seb-MH$_6$-Cws, whereas the slower migrating species represented [^{32}P]protein A. [^{32}P]Phosphoric acid labeling of *S. aureus* RN4220 without pHTT4 (no Seb-MH$_6$-Cws) and immunoprecipitation resulted in the appearance of the 54-kDa species but not of the 44-kDa species (Fig. 3*B*). Furthermore, omission of antibodies from the immunoprecipitation reaction of [^{32}P]phosphoric acid-labeled *S. aureus* RN4220 (pHTT4) failed to generate the two discrete ^{32}P-labeled protein species. Thus, the 44- and the 54-kDa species very likely represent [^{32}P]Seb-MH$_6$-Cws and [^{32}P]protein A, respectively.

[^{32}P]Phosphoric Acid-labeled P3 Intermediates—If the ^{32}P-labeled species reported in Fig. 3*A* represent the P3 interme-

TABLE III
Tunicamycin inhibits the synthesis of surface protein P3 precursor
Data were obtained and quantified from Fig. 7.

Surface protein	^{32}P-Surface protein P3/total cellular ^{32}P concentration			
	5 min[a]	30 min	60 min	120 min
No inhibitor				
Protein A	7	53	36	82
Seb-MH$_6$-Cws	11	46	22	51
Tunicamycin				
Protein A	0	0	0	22
Seb-MH$_6$-Cws	0	0	0	14

[a] Time after the addition of [^{32}P]phosphoric acid to *S. aureus* RN4220 (pHTT4) culture at which labeling was quenched with ice-cold trichloroacetic acid.

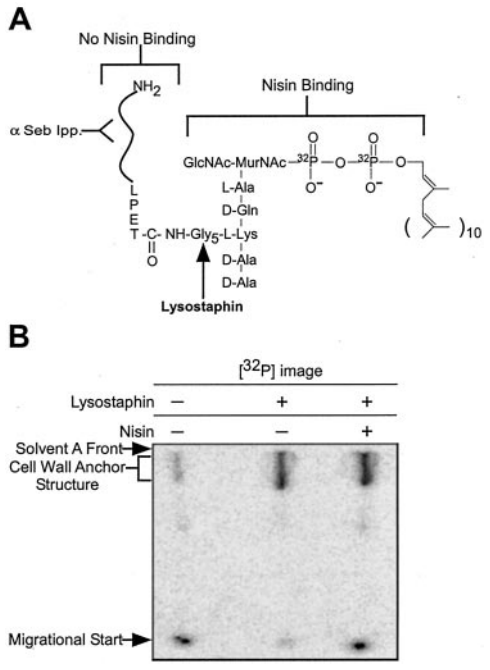

FIG. 8. **Lysostaphin released ^{32}P-labeled anchor species bind to nisin.** Immunoprecipitated ^{32}P-labeled surface protein was either left untreated (mock) or was incubated with lysostaphin. Samples were subjected to thin layer chromatography of K6 silica gel plates using solvent A. After separation for 7 cm, the plates were dried and analyzed by PhosphorImager analysis. In separate reactions, lysostaphin-treated [^{32}P]P3 intermediates were either mock treated or incubated with nisin, a lantibiotic that is known to bind lipid II. Binding of nisin to lipid II prevents migration of the sample on TLC plates.

diate of surface protein anchoring to the cell wall envelope, one can test this assumption in two ways. (i) The structure of this compound (Fig. 1*B*) predicts that treatment of ^{32}P-labeled P3 intermediate with lysostaphin, but not with muramidase, should remove the ^{32}P-label from surface protein. This was tested by first immunoprecipitating surface proteins and then treating the sample with lysostaphin. Lysostaphin digestion converted all [^{35}S]methionine-labeled, muramidase-solubilized surface protein to a faster mobility, consistent with the previously reported removal of C-terminal cell wall anchor structures (12) (Fig. 3*A*). In contrast, lysostaphin digestion of ^{32}P-labeled surface protein removed all radioactive signal from the immunoprecipitated polypeptides (Fig. 3*A*). (ii) The second prediction is that ^{32}P-labeling of P3 precursors should be absolutely dependent on sortase and *S. aureus* mutants lacking *srtA* sortase should fail to incorporate ^{32}P-label into surface protein (Fig. 4). Indeed, [^{32}P]phosphoric acid labeling of *S. aureus* RN4220 (pHTT4) generated the 44- and 54-kDa species, whereas labeling of *S. aureus* SKM1 (*srtA*$^-$) (pHTT4) did not yield a radioactive surface protein signal (Fig. 4 and Table I). Both strains, *S. aureus* RN4220 (pHTT4) and *S. aureus* SKM1 [Δ(*srtA*)] (pHTT4), incorporated equal amounts of [^{32}P]phosphoric acid into cells as judged by liquid scintillation counting of trichloroacetic acid precipitated aliquots. Thus, both of our tests fulfilled the predictions that would be expected for ^{32}P-labeled surface proteins linked to lipid II.

Antibiotic Inhibition of P3 Precursor Synthesis—Several antibiotics are known interfere with the synthesis of the bacterial peptidoglycan. The addition of such compounds may block sortase-mediated biosynthesis of P3 intermediate from P2 precursor and lipid II as substrates. Our experimental approach used *S. aureus* cultures that were treated for 150 min with penicillin, vancomycin, bacitracin, or moenomycin. Vancomycin and moenomycin are known inhibitors of the sorting reaction. Vancomycin binds to the D-Ala-D-Ala moiety of lipid II (28, 29) and prevents both transglycosylase and transpeptidase reactions (30), whereas moenomycin is an inhibitor of transglycosylation alone (31). Penicillin is an inhibitor of the transpeptidation reaction (38). Bacitracin inhibits undecaprenolpyrophosphate dephosphorylation, an essential step in the recycling of lipid II (40). As is shown in Fig. 5*A*, the addition of antibiotics slowed bacterial growth and inhibited bacterial cell wall synthesis (27). Equal numbers of staphylococcal cells from each sample were washed, suspended in minimal medium lacking phosphate, and labeled with [^{32}P]phosphoric acid. To account for the presence of surface protein in each sample, we subjected muramidase-treated samples to SDS-PAGE and immunoblotting with monoclonal antibody α-SPA27 (protein A-specific). As is shown in Fig. 5*B*, treatment of staphylococci with penicillin, vancomycin, bacitracin, or moenomycin led to a significant reduction in the amount of protein A incorporated into the cell wall. Vancomycin and moenomycin caused the largest decrease in surface protein anchoring and display, consistent with previous reports that these antibiotics prevent the incorporation of polypeptides into the cell wall envelope (27, 41).

Treatment of staphylococci with penicillin, vancomycin, or moenomycin abolished the biosynthesis of P3 intermediate (Fig. 6, *A* and *B*). Bacitracin treatment caused a significant reduction in the synthesis of P3 intermediates at all labeling points examined (Fig. 6*B* and Table II). We also tested a known inhibitor of sortase for its effect on the P3 biosynthetic reaction. Staphylococci were treated with MTSET, a reagent that forms disulfide with the active site sulfhydryl of sortase (8). MTSET treatment abolished all P3 biosynthesis. Together these data indicated that the inhibition of both sortase or cell wall biosynthesis prevented P3 precursor formation and the anchoring of surface proteins to the cell wall envelope (27).

Tunicamycin (42), a lipid-linked nucleotide, acts as an inhibitor of phospho-*N*-acetylmuramyl-pentapeptide translocase, an enzyme that synthesizes lipid I (undecaprenolpyrophosphate-MurNAc-L-Ala-D-iGln-(NH$_2$)-L-Lys-D-Ala-D-Ala) from Park's nucleotide (43–45). Tunicamycin treatment is expected to inhibit the formation of the P3 precursor as this antibiotic depletes bacteria of both lipid I and lipid II. Indeed, tunicamycin treatment abolished the formation of ^{32}P-labeled P3 intermediate, strongly supporting the hypothesis that P3 may be composed of surface protein linked to lipid II (undecaprenolpyrophosphate-MurNAc-(L-Ala-D-iGln-(surface protein-Gly$_5$)-L-Lys-D-Ala-D-Ala)-(β1–4)-GlcNAc) (Fig. 7 and Table III).

TLC Analysis of ^{32}P-Labeled Cell Wall Anchor Molecules—Undecaprenyl pyrophosphate is not abundant in staphylococci (less than 1000 molecules in a single bacterium) (46). It seemed improbable that one could achieve isolation of ^{32}P-labeled P3 intermediate in sufficient quantity for mass spectrometry experiments, assuming that the P3 species represents only a

TABLE IV
Thin-layer chromatography of ^{32}P-labeled anchor species released from P3 precursor

Data were generated and analyzed as described in Fig. 8. The ratio of radioactive signals of cell wall anchor species (signal near solvent front) divided by the signal for surface protein P3 intermediate (migrational start) is reported. Nisin forms a complex with lipid II that does not migrate on TLC plates, causing radioactive signals to remain at the start of migrational.

Treatment	[Anchor species]/[P3 intermediate]	
	Solvent A[a]	Solvent B[b]
Mock	0.67	9.8
Lysostaphin	21.80	27.0
Lysostaphin and nisin	2.50	0.636

[a] Isobutyric acid:1 N NH_4OH (5:3, v/v).
[b] *n*-Butanol:acetic acid:water:pyridine (15:3:12:10, v/v).

small fraction of undecaprenol molecules (less than 1%) that are engaged in cell wall and carbohydrate biosynthetic pathways (46). TLC is an alternative method for the analysis of ^{32}P-labeled cell wall anchor structures, because the use of organic solvents on silica plates provides for the separation of lipid but not of larger polypeptides (47). Immunoprecipitated ^{32}P-labeled P3 intermediate was spotted on K6 silica TLC plates, separated with isobutyric acid: 1 N NH_4OH (5:3, solvent A) and analyzed by phosphorimaging. ^{32}P-Labeled P3 intermediate generated a robust radioactive signal (70,000 PhosphorImager counts). Most of the ^{32}P-label was retained at the site of loading, consistent with the expectation that surface proteins cannot migrate with the solvent on the K6 matrix (Fig. 8) (47). Moreover, a second ^{32}P-signal could be detected near the solvent front (Fig. 8).

It was presumed that ^{32}P-labeled anchor species entering the TLC plate may represent lipid II molecules spontaneously released from surface protein. If so, one would predict that the release of ^{32}P-labeled anchor species must be increased by the treatment of surface protein with lysostaphin. This was tested, and lysostaphin treatment greatly diminished the radioactive signal at the migrational start while causing a corresponding increase in the amount of ^{32}P-labeled anchor species. To determine whether or not the ^{32}P-labeled anchor species represent lipid II molecules, we exploited the affinity of the lantibiotic nisin for lipid II (48–50). Previous work showed that the binding of nisin to lipid II resulted in the formation of immobile complexes on TLC (48). Incubation of ^{32}P-labeled anchor species with nisin also resulted in the formation of chromatographically immobile complexes (Fig. 8). This experiment was repeated using two different solvents for the separation of lipids. Solvent A released only small amounts of ^{32}P-labeled cell wall anchor structures from immunoprecipitated surface protein. In contrast, solvent B (*n*-butanol:acetic acid:water:pyridine, 15:3:12:10, v/v) was much more effective in removing ^{32}P-labeled anchor structures from surface protein, even in the absence of lysostaphin treatment. Nisin is known to form a complex with lipid II even under the stringent conditions of solvent B (48). A similar interaction was observed with ^{32}P-labeled anchor species, because the addition of nisin to lysostaphin-digested surface protein led to the formation of immobile, ^{32}P-labeled complexes on TLC (Table IV). In summary, incubation with nisin inhibited the migration of ^{32}P-labeled anchor structures 9-fold in solvent A and 20- to 7-fold in solvent B (Table IV). These data strongly suggest that the ^{32}P-labeled anchor species bind to nisin and, furthermore, that the ^{32}P-labeled P3 intermediate represents surface protein linked to lipid II.

DISCUSSION

Several recent studies focused on characterizing the peptidoglycan substrate of the sortase-catalyzed anchoring reaction. By measuring the processing of pulse-labeled surface proteins, it was determined that both whole cells and staphylococcal protoplasts are capable of anchoring surface proteins (27). Furthermore, antibiotic inhibition of *de novo* bacterial peptidoglycan synthesis inhibits surface protein anchoring (27). Both results are consistent with the view that sortase utilizes the peptidoglycan precursor lipid II but not mature assembled cell walls as substrates for its transpeptidation reaction (2). Another argument in favor of lipid II is the notion that the amide bond between the threonine and the glycine of surface proteins is identical for the substrate (LP*X*TG motif) and the product (LPXT-Gly_5) of the sorting reaction. Thus, if sortase were to interact with assembled peptidoglycan and if sortase catalyzed both forward and reverse transpeptidation reactions, the enzyme would in fact cut cell wall-anchored surface protein. This notion is not supported by our *in vivo* labeling experiments, revealing that sortase rapidly and efficiently anchors surface proteins to the cell wall (3). We presume that surface protein linked to lipid II is rapidly incorporated into the cell wall and that this mechanism prevents sortase from catalyzing a reversible reaction.

Purified sortase catalyzes an *in vitro* transpeptidation reaction of surface protein anchoring using LP*X*TG peptides and NH_2-Gly, NH_2-Gly_2, NH_2-Gly_3, NH_2-Gly_4, or NH_2-Gly_5 as peptidoglycan substrates (26). Previous work used *in vitro* as well as *in vivo* techniques to determine that the pentaglycine cross-bridges (NH_2-Gly_5) are better substrates than shorter cross-bridges for the sorting reaction (11, 26). However, this work still left unresolved whether murein tetra- or pentapeptides with or without linked undecaprenol and disaccharide (lipid II) are the preferred substrate for the *in vitro* sorting reaction. To identify the peptidoglycan substrate of the sorting reaction *in vivo* we entertained the possibility that surface proteins can be labeled with [^{32}P]phosphoric acid to generate the P3 intermediate. Such a species could indeed be observed. The following arguments suggest that P3 intermediates represent surface protein linked to lipid II. (i) ^{32}P-Labeled surface protein (44 kDa) migrates more slowly than the ^{35}S-labeled mature species (30 kDa). The predicted mass of the P3 precursor is about 33 kDa. Assuming that undecaprenolpyrophosphate does not separate on SDS-PAGE in the same manner as polypeptides without lipid decoration, the slower mobility of P3 suggest that lipid is indeed attached to surface protein. (ii) The ^{32}P-label of surface proteins can be removed by lysostaphin digestion but not by muramidase treatment. (iii) The formation of ^{32}P-labeled P3 precursor absolutely requires sortase as treatment with MTSET or deletion of the sortase gene abolishes its appearance. (iv) Inhibition of peptidoglycan synthesis with antibiotics interferes with the biosynthesis of ^{32}P-labeled P3 precursor. (v) Lysostaphin treatment of P3 intermediates results in the release of ^{32}P-labeled anchor species that can be separated on TLC plates. (vi) Nisin, a lantibiotic that is known to bind lipid II, also forms a complex with ^{32}P-labeled anchor species. Together these data suggest that ^{32}P-labeled P3 precursor likely represents surface protein linked to lipid II. It is anticipated that P3 intermediates are present in very small numbers in living cells, because lipid I and lipid II molecules are the least abundant intermediates of peptidoglycan biosynthesis (46). It seems certain that the purification and analysis strategies established for surface proteins anchored to cell wall can not be used for the isolation of P3 (10).

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