Surface proteins of Staphylococcus aureus are covalently linked to the bacterial cell wall by a mechanism requiring a COOH-terminal sorting signal with a conserved LPXTG motif. Cleavage between the threonine and the glycine of the LPXTG motif liberates the carboxyl of threonine to form an amide bond with the amino of the pentaglycine cross-bridge in the staphylococcal peptidoglycan. We asked whether antibiotic cell wall synthesis inhibitors interfere with the anchoring of surface proteins. Penicillin G, a transpeptidation inhibitor, had no effect on surface protein anchoring, whereas vancomycin and moenomycin, inhibitors of cell wall polymerization into peptidoglycan strands, slowed the sorting reaction. Cleavage of surface protein precursors did not require a mature assembled cell wall and was observed in staphylococcal protoplasts. A search for chemical inhibitors of the sorting reaction identified methanethiosulfonates and \( p \)-hydroxymercuribenzoic acid. Thus, sortase, the enzyme proposed to cleave surface proteins at the LPXTG motif, appears to be a sulfhydryl-containing enzyme that utilizes peptidoglycan precursors but not an assembled cell wall as a substrate for the anchoring of surface protein.

To infect and multiply within their human hosts, Gram-positive bacteria require surface proteins that either bind to specific organ tissues or provide ingenious strategies for bacterial escape from the immune response (1). The mechanism of surface protein anchoring to the bacterial cell wall has recently been established for protein A of Staphylococcus aureus. After synthesis in the cytoplasm, protein A is initiated into the secretory pathway by an NH\(_2\)-terminal leader peptide (2). A COOH-terminal sorting signal is necessary and sufficient for the anchoring of protein A and functions first to retain the carboxyl of D-Ala at position four is amide-linked to the amino of the pentaglycine cross-bridge (11). Elements involved in transpeptidation and cell wall sorting are conserved, suggesting that surface protein anchoring is a universal mechanism in Gram-positive pathogens (1, 12). If so, sortase, the enzymatic activity that is thought to catalyze this reaction, might also be found conserved in many different bacterial species. Methods that inhibit sortase may be used as anti-bacterial therapies for the treatment of human infections caused by Gram-positive microbes.

Although surface protein anchoring has been characterized in molecular detail, little is known about the peptidoglycan substrate. Because a biochemical in vitro reaction for sortase has not yet been established, we approached this question by searching for inhibitors of cell wall sorting. During bacterial cell wall synthesis, a soluble cytoplasmic peptidoglycan precursor (UDP-MurNac-L-Ala-D-\( \gamma \)-Gln-D-Lys-D-Ala-D-Ala), where D-Gln is iso-glutaminy1; Park’s nucleotide) is linked to undecaprenylpyrophosphate to generate lipid I (13–15). This membrane-bound intermediate is further modified by the addition of GlcN and pentaglycine (undecaprenylpyrophosphate-MurNac-L-Ala-D-\( \gamma \)-Gln-(NH\(_2\)-Gly\(_5\))-L-Lys-D-Ala-D-Ala) (\( \beta \)-1–4)-GlcNac; also named lipid II) and then translocated across the cytoplasmic membrane (14, 16, 17). Lipid II serves as a substrate for the transglycosylation reaction, which polymerizes the glycan strands of the bacterial cell wall to yield repeating disaccharide (MurNac-GlcNac\(_n\), (18). Cell wall pentapeptides (L-Ala-D-\( \gamma \)-Gln-L-Lys-D-Ala-D-Ala) linked to the MurNac of nascent peptidoglycan strands are cross-linked via the transpeptidation reaction, thereby generating a three-dimensional cell wall network that protects bacteria from osmotic lysis (11, 19).

Penicillin is an inhibitor of the transpeptidation reaction (20). This class of \( \beta \)-lactam antibiotics functions as a molecular mimicry of D-Ala-D-Ala (11) and, after cleavage of the antibiotic by transpeptidases (penicillin-binding proteins), continues to occupy their active site serine (21). Although penicillin effectively blocks the cross-linking of wall peptides (19), it does not interfere with the transglycosylation reaction (22). Vancomycin binds to the D-Ala-D-Ala moiety of lipid II (23, 24), thereby preventing substrate recognition by penicillin-binding proteins that catalyze both transglycosylation and transpeptidase reactions (11, 25). Moenomycin is an inhibitor of transglycosylation because this compound inhibits C\(_{5,5}\) isoprenoid-alcohol kinase (26, 27) as well as the transglycosylase activity of penicillin-binding proteins (28). Here we report that vancomycin and moenomycin, but not penicillin, cause a reduction in the rate of surface protein anchoring, suggesting that sortase may utilize lipid II as a substrate for the sorting reaction. Consistent with this hypothesis is our observation that staphylococcal protoplasts, in which the assembled cell wall has been removed by digestion with muralectam enzyme, catalyze the cleavage of surface proteins at their LPXTG motif similar to bacteria with an intact cell wall. A search for chemical inhibitors of the sorting reaction identified methanethiosulfonates as well as organic

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mercurials, indicating that sortase must be a sulfhydryl-containing enzyme.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—**Plasmids pSeb-Spa<sub>490–524</sub> (3), pSeb-Cws-Blaz, and pSeb-Cws<sub>LPXTG</sub>-BlaZ (5) were transformed into *S. aureus* OS2<sub>1</sub> (spa<sub>ermC</sub>, r<sup>−</sup>) (2) and have been described previously. Staphylococci were generally grown in tryptic soy broth or agar. All chemicals were purchased from Sigma unless indicated otherwise.

**Characterization of Cell Wall Sorting Intermediates—** *S. aureus* OS2<sub>2</sub>pSeb-Spa<sub>490–524</sub> was grown overnight in CDM (29) (Joel Biosciences) supplemented with chloramphenicol (10 μg/ml), diluted 1:10 into minimal medium, and grown with shaking at 37°C until *A<sub>600</sub>* 0.6. Cells were labeled with 100 μCi of <sup>35</sup>S-labeled Promix (Amersham Pharmacia Biotech) for 1 min. Labeling was quenched by the addition of an excess nonradioactive amino acid (50-μl chase (100 mg/ml casamino acids, 20 mg/ml methionine and cysteine)). At timed intervals (0, 1, 3, and 10 min) after the addition of the chase, 250-μl aliquots were removed, and proteins were precipitated by the addition of 250 μl of 10% trichloroacetic acid. The precipitate was sedimented by centrifugation at 15,000 g for 10 min, washed with 1 ml of acetone, and dried. Samples were suspended in 1 ml of 0.5 M Tris-HCl, pH 6.8, and staphylococcal peptidoglycan was digested by adding 50 μl of lysozymin (30) (100 μg, AMBI Pharmaceuticals) and incubating for 1 h at 37°C.

Proteins were again precipitated with trichloroacetic acid, washed with acetone, and subjected to immunoprecipitation with α-Seb<sup>1</sup> followed by SDS-PAGE and PhosphorImager analysis. To characterize the P1 and P2 precursors, 1 ml of culture was either incubated with 5 mM sodium azide or no azide prior to labeling or 5 mM MTSET was added 15 s after the beginning of the pulse.

**Antibiotic Inhibition of Cell Wall Sorting—** Overnight cultures of *S. aureus* OS2<sub>2</sub>pSeb-Spa<sub>490–524</sub> grown in CDM were diluted into fresh minimal medium and incubated until *A<sub>600</sub>* 0.3. Cultures were then treated with penicillin (10 μg/ml), vancomycin (10 μg/ml), or moenomycin (10 μg/ml) or were left untreated. A 0.5-ml culture sample was removed for pulse labeling with 100 μCi of <sup>35</sup>S-labeled Promix for 5 min. Labeling was quenched and proteins precipitated by the addition of 0.5 ml of 10% trichloroacetic acid. The precipitate was collected by centrifugation, washed in acetone, and dried under vacuum. The pellets were suspended in 1 ml of 0.5 M Tris-HCl, pH 7.0, 50 μl of lysozymin (100 μg/ml, AMBI Pharmaceuticals) was added, and the staphylococcal cell wall was digested by incubating for 1 h at 37°C. Proteins were precipitated with trichloroacetic acid, washed in acetone, dried, solubilized in 50 μl of 0.5 M Tris-HCl, pH 7.5, 4% SDS, and boiled for 10 min. Aliquots of solubilized surface protein were immunoprecipitated with α-Seb<sup>1</sup> followed by SDS-PAGE and PhosphorImager analysis.

**Peptidoglycan Synthesis Measurements—**Staphylococci were grown in the presence or absence of antibiotics as described above. At 30-min intervals, 0.5-ml culture samples were withdrawn and labeled with either 50 μCi of [<sup>3</sup>H]lysine or 50 μCi of [<sup>14</sup>Cl]lysine for 20 min (31). All labeling was quenched by the addition of 0.5 ml of 20% trichloroacetic acid. Samples were heated to 96°C for 30 min, cooled to room temperature, and pipetted onto glass fiber filters. The filters were placed in a holder and washed under vacuum suction with 25 ml of 75% ethanol and 4 ml of distilled water.

**Chemical Inhibitors of the Sorting Reaction—** *S. aureus* OS2<sub>2</sub>pSeb-Spa<sub>490–524</sub> was grown overnight in CDM supplemented with chloramphenicol (10 μg/ml), diluted 1:10 into minimal medium, and grown with shaking at 37°C until *A<sub>600</sub>* 0.6. Cells were labeled with 100 μCi of <sup>35</sup>S-labeled Promix for 5 min. Chemicals were added to a final concentration of 5 mM 15 s after the beginning of the pulse. All labeling was quenched by adding trichloroacetic acid to 10%. Precipitated cells and proteins were collected by centrifugation, washed in acetone and the staphylococcal cell wall digested with lysozymin as described above. The digests were again precipitated with trichloroacetic acid, immunoprecipitated with anti-α-Seb antibodies, and subjected to SDS-PAGE and PhosphorImager analysis.

**RESULTS**

**Inhibitors of the Cell Wall Sorting Reaction—** We sought to identify compounds that interfered with the anchoring of surface proteins by testing known inhibitors of proteases. Our experimental design employed the reporter protein Seb-Spa<sub>490–524</sub> harboring an NH<sub>2</sub>-terminal leader (signal) peptide with a signal peptidase cleavage site as well as a COOH-terminally fused cell wall sorting signal consisting of the LPXTG motif, hydrophobic domain (shaded box), and positively charged tail (boxed RRREL). B, cell wall sorting of Seb-Spa<sub>490–524</sub> was observed by pulse labeling staphylococcal cultures with [<sup>35</sup>S]methionine for 1 min and quenching all further incorporation by the addition of excess unlabeled methionine (chase). At the indicated time intervals, aliquots of labeled culture were withdrawn, and proteins were precipitated with trichloroacetic acid and washed in acetone. The precipitate was suspended, and the staphylococcal cell wall was digested with lysozymin. Protein was again precipitated with trichloroacetic acid followed by immunoprecipitation with α-Seb. Samples were separated on 14% SDS-PAGE, and processing of Seb-Spa<sub>490–524</sub> was quantified by PhosphorImager analysis. Export of the leader peptide bearing P1 precursor from the bacterial cytosol was inhibited by adding 5 mM sodium azide (+Na<sub>3</sub>N) 5 min prior to labeling. Cleavage of the leader peptide-less P2 precursor between the threonine and the glycine of the LPXTG motif was inhibited by the addition of methaneethiol-fonate (+MTSET) 15 s after the beginning of the pulse. Mature Seb-Spa<sub>490–524</sub> is linked to the cell wall via an amide bond between the carbonyl of threonine and the amino of the pentaglycine cross-bridge.

precipitated with α-Seb followed by SDS-PAGE and PhosphorImager analysis.

**Cell Wall Sorting in Staphylococcal Protoplasts—** Overnight cultures of *S. aureus* OS2<sub>2</sub>pSeb-Cws-Blaz* or S. aureus OS2<sub>2</sub>pSeb-Cws<sub>LPXTG</sub>Blaz* grown in CDM were diluted 1:10 into minimal medium and grown with shaking at 37°C until *A<sub>600</sub>* 0.6. One ml of culture was pulse-labeled with 100 μCi of <sup>35</sup>S-labeled Promix for 2 min, and labeling was quenched by the addition of 50 μl of chase solution. Culture aliquots (0.5 ml) were removed for trichloroacetic acid precipitation either during the pulse or 20 min after the addition of chase. Another culture aliquot was first converted to protoplasts and then subjected to labeling. The cells were sedimented by centrifugation at 15,000 x g for 5 min and suspended in 1 ml of 50 mM Tris-HCl, 0.4 M sucrose, 10 mM MgCl<sub>2</sub> pH 7.5. Protoplasts were washed in sucrose buffer and labeled with 100 μCi of <sup>35</sup>S-labeled Promix for 2 min, and labeling was quenched by the addition of 50 μl of chase solution. For sedimentation analysis, pulse-labeled protoplasts were centrifuged at 15,000 x g for 10 min to separate soluble surface proteins from those that were bound to cell membranes. All samples were precipitated with trichloroacetic acid, washed in acetone, and suspended in 50 μl of 4% SDS, 0.5 M Tris-HCl, pH 7.5, with boiling for 10 min. Aliquots of solubilized surface protein precursor and anchored products were immunoprecipitated with α-Seb and α-Blaz and subjected to SDS-PAGE and PhosphorImager analysis.

**FIG. 1. Inhibitors of surface protein export and anchoring to the staphylococcal cell wall.** A, structure of Seb-Spa<sub>490–524</sub> harboring an NH<sub>2</sub>-terminal leader (signal) peptide with a signal peptidase cleavage site as well as a COOH-terminally fused cell wall sorting signal consisting of the LPXTG motif, hydrophobic domain (shaded box), and positively charged tail (boxed RRREL).
all three species, P1 and P2 precursors as well as mature Seb-Spa490–524, were detected (Fig. 1). Within 1 min after the addition of the chase, most pulse-labeled surface protein was converted to the mature, anchored species. Surface protein anchoring was complete 3 min after the quenching of [35S]methionine incorporation.

Sodium azide is an inhibitor of SecA, an essential component of the secretory pathway in bacteria (32). Addition of 5 mM sodium azide to staphylococcal cultures 5 min prior to pulse-labeling significantly reduced protein export and led to the accumulation of a leader peptide bearing P1 precursor (2) (Fig. 1). Methanosulfonates react with sulfhydryl groups (33), and one of these compounds, [2-(trimethylammonium)ethyl]methanosulfonate (MTSET), prevented incorporation of [35S]methionine by staphylococci. However, when added 15 s after the beginning of the pulse, MTSET interfered with the cleavage of sorting signals at the LPXTG motif, whereas the Sec-dependent export of the P1 precursor remained unaltered. This result revealed that sortase must harbor a sulfhydryl group that is necessary for enzymatic cleavage at the LPXTG-bearing sorting signals.

If sortase requires sulphydryl for enzymatic activity, the addition of other sulphydryl reagents may also inhibit the cleavage of sorting signals at the LPXTG motif. This assumption was tested, and (2-sulfonatoethyl)methanosulfonate (MTSET), another methanosulfonate, also interfered with sorting, albeit not as effectively as MTSET (Table I). Furthermore, p-hydroxymercureibenzoic acid, an organic mercurial known to inhibit cysteine proteases, displayed an inhibitory effect, whereas alkylating reagents such as N-ethylmaleimide, iodoacetamide, and iodoacetamide did not (34). Sulphydryl reducing agents, i.e., dithiothreitol and mercaptoethanol, did not affect the sorting reaction. Neither phenylmethylsulfonyl fluoride, which reacts with hydroxyl (34), nor treatment with the divalent cation chelator EDTA interfered with cell wall sorting, indicating that sortase likely does not require divalent cations or hydroxyl for cleavage and anchoring of surface protein.

Antibiotic Inhibition of Bacterial Cell Wall Synthesis and Cell Wall Sorting—To examine the effect of known antibiotics on cell wall sorting, we chose three compounds, penicillin, vancomycin, and moenomycin. S. aureus OS2 (pSeb-Spa490–524) was grown in minimal medium to Amax 0.3, treated with 10 μg/ml penicillin, vancomycin, or moenomycin, and incubated for an additional 5 h (Fig. 2). At 30-min intervals during this experiment, aliquots were withdrawn for measurements of surface protein sorting and cell wall synthesis. The effect of antibiotics on the rate of bacterial cell wall synthesis was determined as the ratio of [3H]lysine/[3H]leucine label incorporated into acid-precipitable, Pronase-resistant peptidoglycan (31). Lysine is a component of peptidoglycan, whereas leucine is not. Hence, the ratio of incorporation of these two amino acids is a measure of cell wall synthesis. Surface protein anchoring was measured by pulse labeling and quantified as the ratio between the concentration of P2 precursor [P2] and mature, anchored Seb-Spa490–524 [M]. The addition of vancomycin, penicillin, or moenomycin reduced the growth rate of staphylococci as compared with a mock-treated control. Whereas the rate of cell wall sorting precursor cleavage remained constant during the growth of mock-treated staphylococci, the addition of vancomycin led to a steady accumulation of P2 precursor, indicating that this compound caused a reduction of the sorting reaction. A similar, albeit weaker, effect was observed when moenomycin was added to staphylococcal cultures. In contrast, penicillin G did not alter the rate of cell wall sorting. As expected, all three antibiotics diminished the rate of peptidoglycan synthesis (Table II). Together these data revealed that vancomycin and moenomycin cause a reduction in the rate of cell wall sorting, whereas penicillin has no effect on surface protein anchoring.

Cell Wall Sorting in Staphylococcal Protoplasts—Previous work revealed that protoplasts, generated by murapectic digestion of staphylococci or penicillin selection of streptococcal L
forms, secrete surface protein into the surrounding medium (35, 36). This observation can be explained in two ways. Either the COOH-terminal sorting signals cannot retain surface proteins in the envelope of protoplasts, or the presence of an intact, assembled cell wall is not required to cleave sorting signals at their LPXTG motif. To distinguish between these possibilities, we measured surface protein anchoring in intact bacteria and staphylococcal protoplasts (Fig. 3). Wild-type staphylococci cleaved the Seb-Cws-BlaZ precursor to generate the mature, anchored NH2-terminal Seb and COOH-terminal cytoplasmic BlaZ fragments (5). When tested in staphylococcal protoplasts generated by lysostaphin digestion of the cell wall, precursor cleavage occurred similar to that in whole cells, indicating that the presence of a mature, assembled cell wall is not required for cleavage of sorting signals. We observed unique sorting products in protoplasts that migrated more slowly than mature, anchored Seb (see arrow in Fig. 3). As these species were immunoprecipitated with α-Seb but not with α-BlaZ (data not shown), they likely represent products of the sorting reaction. The COOH-terminal anchor structure of the protoplast species must be distinct from that generated by lysostaphin digestion of anchored Seb (three glycyl attached to the carboxyl of threonine), because the proteins migrated more slowly on SDS-PAGE than lysostaphin-released Seb.

To examine whether all cleaved Seb fragments were released into the extracellular medium, pulse-labeled protoplasts were sedimented by centrifugation and separated from the extracellular medium in the supernatant. All Seb-Cws-BlaZ precursor and COOH-terminal BlaZ cleavage fragments sedimented with the protoplasts. In contrast, NH2-terminal Seb fragments that migrated at the same speed as Seb released by lysostaphin digestion from the cell wall of intact staphylococci were soluble in the culture medium. Some, but not all, of the more slowly migrating Seb species sedimented upon centrifugation, suggesting that these products of the sorting reaction may be attached to protoplast membranes. No precursor cleavage was observed for Seb-CwsLPXTG-BlaZ either in whole cells or staphylococcal protoplasts.

**DISCUSSION**

To characterize the peptidoglycan substrate of the cell wall sorting reaction, we performed two experiments. If cell wall sorting requires the pentaglycine cross-bridges of mature, assembled cell wall as a substrate, we would expect that the treatment of bacterial cultures with antibiotics should not affect the anchoring of surface proteins. However, both moenomycin and vancomycin treatment of staphylococci slowed the sorting reaction. Moenomycin is an inhibitor of transglycosylation, whereas vancomycin binds to D-Ala-D-Ala within lipid II. Both moenomycin and vancomycin are known to prevent incorporation of lipid II precursors into peptidoglycan (26, 37). Inhibition of cell wall sorting was increased with prolonged incubation of staphylococci in the presence of antibiotic, suggesting that moenomycin and vancomycin do not interfere directly with the sorting reaction. Rather, these compounds alter the physiologic concentration of peptidoglycan precursor molecules that may serve as a substrate for the sorting reaction (22). If so, penicillin G, which inhibits the cross-linking of polymerized peptidoglycan strands without altering the concentration of lipid II (22), should not affect the sorting reaction. This predicted result was indeed observed. Thus, we suggest that the observed inhibition of surface protein anchoring by moenomycin and vancomycin may be the result of the reduced availability of lipid II for the sorting reaction.

The question of antibiotic inhibition of anchoring protein A to the cell wall has been addressed previously. Although the mechanism of cell wall sorting was unknown at the time, Mo-
Cell Wall Sorting Inhibitors

vitz (38) examined the effect of vancomycin on the incorporation of protein A into cell wall polymer and observed no effect. In contrast to our measurements of the rate of cell wall sorting precursor cleavage, these observations relied on the binding of cell wall-associated protein A to immunoglobulin as a measure of the amount of surface protein anchoring. We think it is likely that the discrepancy between Movitz’ results and our data may be related to the nature of the assays employed to measure cell wall anchoring of protein A. If sortase, the enzyme that catalyzes cleavage of surface proteins at the LPXTG motif and linkage to peptidoglycan cross-bridges, does not require mature, assembled cell wall, cleavage of precursors should proceed in staphylococcal protoplasts. This prediction was confirmed here. Furthermore, we also observed cleaved surface protein with unique mobility on SDS-PAGE. Some of these species appeared to sediment with the protoplast membranes, an observation that is consistent with the linkage of surface protein to lipid-linked peptidoglycan precursor (27). At this time, we cannot provide biochemical proof for the existence of lipid-linked sorting intermediates because such molecules have not yet been purified and characterized.

Sortase functions as a transpeptidase, and simple hydrolysis of the sorting signal by cleavage through the threonine and the glycine of the LPXTG motif has never been observed (1). Transpeptidation can be accomplished via an active site hydroxyl or sulfhydryl of sortase, which may thereby capture surface proteins as acyl enzyme intermediates (39). To distinguish between the possibilities of an active site hydroxyl or sulfhydryl, we searched for inhibitors of the sorting reaction and report that cleavage at the LPXTG motif of surface proteins is sensitive to methanethiosulfonates and p-hydroxymercuribenzoic acid. Thus, it appears that sortase utilizes a sulfhydryl group to form an acyl intermediate with cleaved polypeptide chains. This hypothesis is supported by two observations. First, several slower reacting sulfhydryl alkylating reagents do not interfere with sorting, consistent with the notion that the active site sulfhydryl of sortase is generally occupied as an acyl enzyme. Second, recent isolation of the structural gene for sortase revealed the presence of a single cysteine (sulfhydryl group) that is conserved in homologous enzymes identified by data base searches in the genomes of other Gram-positive bacteria.2 3

Pancholi and Fischetti (40) described a membrane anchor cleavage enzyme activity of M protein when Streptococcus pyogenes was pretreated with muralytic enzyme. The chemical nature of this M protein cleavage and release into the extracellular milieu of protoplasts is not known. Although membrane anchor cleavage enzyme can be inhibited by organic mercurials such as p-hydroxymercuribenzoic acid, this activity is sensitive to the addition of divalent cations (40). Thus, membrane anchor cleavage enzyme must be distinct from sortase, as this enzyme cannot be inhibited by either ZnCl2 or CaCl2.

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