Surface proteins of the Gram-positive organism *Staphylococcus aureus* are anchored to the bacterial cell wall by a transpeptidation mechanism during which the polypeptide is cleaved between the threonine (T) and the glycine (G) of the LPXTG motif. The carboxyl of threonine is subsequently amide linked to the amino of the pentaglycyl cross-bridge within the staphylococcal peptidoglycan. Previous work examined the anchor structure of surface proteins solubilized from the peptidoglycan by treatment with lysostaphin or *Φ11* hydrolyase and identified COOH-terminally linked triglycyl or L-Ala-ιGln-L-Lys(Gly₃)-ιAla and MurNAc-L-Ala-ιGln-L-Lys(Gly₃)-ιAla[(β1–4)-GlcNAc], respectively. Here, we report the anchor structure of surface proteins solubilized with *N*-acytelymuramidase and *N*-acytelymuramyl-L-alanine amidase. *N*-Acetylmuramidase-released surface protein was linked to MurNAc-L-Ala-ιGln-L-Lys(Gly₃)-ιAla[(β1–4)-GlcNAc], whereas *N*-acytelymuramyl-L-alanine amidase treatment of the cell wall solubilized surface proteins linked to L-Ala-ιGln-L-Lys(Gly₃)-ιAla. Most, but not all, anchor structures were cross-linked to other cell wall subunits, in which the n-alanyl at position four was amide linked to the pentaglycyl of a neighboring wall peptide.

Proteins displayed on the surface of *Staphylococcus aureus* are covalently linked to the peptidoglycan by a mechanism requiring a COOH-terminal sorting signal (1). Previous work on staphylococcal protein A revealed that the 35-residue sorting signal is composed of an LPXTG motif, a hydrophobic domain, and a COOH-terminal tail of mostly positively charged residues (1). Sorting signals with similar structure have been identified in many surface proteins of several different Gram-positive species (2, 3). When expressed in *S. aureus*, these sorting signals function to anchor hybrid reporter proteins to the peptidoglycan (2). Thus, surface protein anchoring to the bacterial cell wall appears to be a universal mechanism in Gram-positive organisms.

Bacterial cell wall (murein) is a heteropolymer containing glycan and peptide components (4). The glycan chains consist of a repeating disaccharide, *N*-acytelymuramyl-(β1–4)-*N*-acytelyglucosamine (MurNAc-GlcNAc)³ (5). The lactyl of *N*-acytelymuramyl is amide linked to the amino of L-alanyl within the wall peptide (NH₂-L-Ala-ιGln-L-Lys-D-Ala-D-Ala-COOH) (6–9). Wall peptides of neighboring peptidoglycan strands can be cross-linked by a transpeptidation mechanism during which the terminal d-alanine is cleaved, and the liberated carboxyl of D-alanyl at position four is amide linked to the free amino of the cell wall cross-bridge (10). In staphylococci, the cross-bridge consists of five glycyl linked to the e-amino of lysyl in the wall peptide [MurNAc-L-Ala-ιGln-L-Lys(NH₂-Gly₃)-ιAla-ιAla-ιAla-COOH][(β1–4)-GlcNAc] (11, 12). Other bacteria employ either the e-amino itself or incorporate other amino acids into the cross-bridge (13). Cross-linking, i.e., the transpeptidation reaction, is catalyzed by penicillin binding proteins and can be inhibited by *β*-lactam antibiotics (6, 14, 15). About 99% of wall peptides within the staphylococcal cell wall are cross-linked (12).

Covalent attachment of surface proteins to the staphylococcal cell wall also occurs by a transpeptidation mechanism, whereby the sorting signal is cleaved between the threonine (T) and the glycine (G) of the LPXTG motif upon export of the polypeptide from the bacterial cytoplasm (16). The carboxyl of threonine is subsequently amide linked to the pentaglycyl cross-bridge of the staphylococcal cell wall (17). Previous work investigated the anchor structure of surface proteins that were released from the cell wall of *S. aureus* by enzymatic cleavage of the peptidoglycan at unique sites. Lysostaphin cut at the pentaglycyl cross-bridge and released surface protein with two or three glycyl linked to the carboxyl of threonine (T) in the LPXTG motif (17, 18). Digestion with the murein hydrolase of staphylococcal phage *Φ11* released surface protein as two species, one of which contained the branched anchor peptide NH₂-L-Ala-ιGln-L-Lys(Gly₃)-ιAla-COOH linked to the carboxyl of threonine, whereas the other harbored an additional disaccharide moiety (MurNAc-L-Ala-ιGln-L-Lys(NH₂-Gly₃)-ιAla-D-Ala-ιAla-COOH)[(β1–4)-GlcNAc] (18).

Analysis of the predicted amino acid sequence of *Φ11* hydrolase revealed homology to known *N*-acytelymuramyl-L-alanine amidasases (19). Assuming the *Φ11* enzyme functioned as an *N*-acytelymuramyl-L-alanine amidasase, we previously proposed that staphylococcal surface proteins may be linked to unsubstituted (non-cross-linked) cell wall tetrapeptide (18). This interpretation is in disagreement with the observed high degree of cross-linking of staphylococcal peptidoglycans (12, 20–22).

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³To whom reprint requests should be addressed: Dept. of Microbiology and Immunology, UCLA School of Medicine, 10833 Le Conte Ave., Los Angeles, CA 90095. Tel.: 310-206-0997; Fax: 310-267-0173; E-mail: olaf@ucla.edu.

1 The abbreviations used are: MurNAc, *N*-acytelymuramic acid; CH₃CN, acetonitrile; CID, collisionally induced dissociation; Cws, cell wall sorting signal; ESI-MS, electrospray ionization mass spectrometry; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; MS/MS, tandem mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; PPAG, polypropylene glycol; RP-HPLC, reversed phase high performance liquid chromatography; Seb, staphylococcal enterotoxin B.
To address this issue, we purified and characterized anchor peptides of surface proteins that were released from the staphylococcal peptidoglycan by treatment with mutanolysin, an N-acetylmuramidase of Streptomyces globisporus (23, 24), as well as two other N-acetylmuramy-l-alanine amidases, autolysin (Atl) of S. aureus (25) and cell wall lysozyme (CwlA) of Bacillus subtilis, respectively (26–28). The data revealed that surface proteins were linked mostly to cross-linked cell wall tetrapeptide. We present a refined model of the cell wall anchor structure that accounts for the observed solubilization patterns of surface proteins with murin hydroxylases.

**EXPERIMENTAL PROCEDURES**

**Strains and Materials—**S. aureus OS2 (pHTT4) was employed for all preparations of staphylococcal cell wall with linked Seb-MH-Cws (18). Escherichia coli XL-1-Blue (pWil-50) was used for the expression of CwlA (29). The cloning sequence of the B. subtilis cwaA amide (27) was polymerase chain reaction amplified from B. subtilis MN2022 chromosomal DNA using the primers BSA-1 (AAAGATCTTAAATCTGAAATCGTAT- TA) and BSA-2 (AACCCTGATTCATTTGGAACCTGTTA). The polymerase chain reaction product was digested with BamHI and PstI and ligated into the corresponding sites of pQE30 (Qiagen). The plasmid pWi150. Tryptic soy broth (TSB) was purchased from Difco. Mutanolysin (Sigma) and lysostaphin (Ambo, UK) were purchased and used as recommended by the supplier.

**Preparation of Staphylococcal Cell Walls—**6 liters of S. aureus OS2 (pHTT4) culture were grown overnight in tryptic soy broth (10 μg/ml chloramphenicol). The cells were harvested by centrifugation at 10,000 × g for 15 min, and the supernatant was discarded. Staphylococci were washed in 50 ml of 50 mM Tris-HCl, pH 7.5, and suspended in the same buffer supplemented with 1 μg pepstatin and 5 mM PMSF. Cell walls were broken with a Bead-Beater instrument ( Biospec Products; Bartlesville, OK) by 15 pulses of 40 s each followed by a 5-min incubation period on ice. The lysate was cleared by ultracentrifugation at 32,500 × g for 15 min, and the supernatant was discarded. The sediment was suspended in 50 ml of wash buffer (100 mM potassium phosphate, pH 7.5, 1% Triton X-100, 1 mM PMSF, and 1 μg pepstatin), and membrane detergent was extracted by stirring for 3 h at 4 °C. The cell walls were sedimented by centrifugation for 15 min at 32,500 × g, washed three times with 100 mM sodium phosphate, pH 6.0, and once with water, and pellets were stored at −20 °C.

**Solubilization and Purification of Surface Proteins—**Mutanolysin was suspended at a concentration of 5,000 units/ml in 100 mM sodium phosphate, pH 6.0, containing 1 mM PMSF and stored at −20 °C. Cell walls were suspended in 100 mM sodium phosphate buffer, usually 30 μl per peptidoglycan from 6 liters of culture; 10,000 units of mutanolysin was added, and the lysis reaction was incubated for 16–18 h at room temperature in the dark. Samples were dried under vacuum and washed twice with 0.5 ml of water. Pellets were dissolved in 1–2 ml of buffer A (6 mM guanidine hydrochloride, 0.1 μM NaHPO₄, 0.01 mM Tris-HCl, pH 8.0) and loaded onto a column packed with 1 ml of Ni-NTA Sepharose pre-equilibrated with 10 ml of buffer A. The column was washed with 10 ml of each buffer A, buffer B (8 M urea, 100 mM NaHPO₄, 0.01 mM Tris-HCl, pH 8.0), and buffer C (same as buffer B, but pH 6.3). Peptides were eluted with 2 ml of buffer D (6 M guanidine hydrochloride, 0.5 mM acetic acid, pH 4.3) and subjected to RP-HPLC on C18 column (2 × 250 mm, C18 Hypersil, Keystone Scientific). Separation was carried out at 40 °C with a flow rate of 0.2 ml/min and a linear gradient starting 10 min after injection from 99% H₂O, 0.1% trifluoroacetic acid to 60% H₂O, 0.1% trifluoroacetic acid, and 40% CH₃CN in 35 min followed by a steep gradient to 99% CH₃CN in 5 min. Eluate absorbance was monitored at 215 nm, and 1-min fractions were collected.

**MALDI-MS—**Dried HPLC fractions were suspended in CH₃CN:water:trifluoroacetic acid (50:50:0.1), typically 50 μl per 1.5 absorbance unit. MALDI mass spectra were obtained on a reflectron time-of-flight instrument (PerSeptive Biosystems Voyager LR) in the linear mode. Samples (0.5 μl) were co-spotted with 0.5 μl of matrix (α-cyano-4-hydroxy-cinnamic acid, 10 mg/ml in CH₃CN:water:TFA (70:30:0.1)). All samples were externally calibrated to a standard of bovine insulin.

**HPLC Separation of Muropeptides—**Anchor peptides solubilized with either Gst-Atl or mutanolysin were reduced by treatment with sodium borohydride (33). After HPLC purification on C18 column, anchor peptides were dried and suspended in 200 μl of water. To the peptides, 200 μl of 0.5 mM sodium borate buffer, pH 9.0, was added followed by the immediate addition of 1–3 mg of solid sodium borohydrate. The reaction was incubated for 30 min at room temperature and quenched by the addition of 20 μl of 20% phosphoric acid. Reduced peptides were desalted over a C18 cartridge (Analytichem), dried under vacuum, and suspended in 50 mM Tris-HCl, pH 7.5. The glycyl-glycine endopeptidase lysostaphin (Ambi) was added at a concentration of 200 μg/ml and incubated overnight at 37 °C. Lysostaphin digestion was terminated by the addition of trifluoroacetic acid to a concentration of 10%. Samples were placed on ice to precipitate lysostaphin, which was separated from the soluble muropeptides by centrifugation at 15,000 × g for 15 min. Separation of muropeptides by RP-HPLC on C18 column (2 × 250 mm, C18 Hypersil, Keystone Scientific) was carried out using a method devised for E. coli wall peptides (33, 34) and modified for the separation of S. aureus muropeptides (35). Briefly, peptides were chromatographed by a linear reversed phase gradient from 5% (v/v) methanol containing 100 mM NaHPO₄ (pH 2.5) to 30% (v/v) methanol in 100 mM NaHPO₄ (pH 2.8) in 100 min. Baseline detection was by subtracting the chromatogram of a blank run. Eluate absorbance was monitored at 206 nm, and peak fractions were desalted using a C18 cartridge and dried under vacuum prior to analysis by ESI-MS.

**ESI-MS of Anchor Peptides—**Dried muropeptides were dissolved in 30 μl of water:CH₃CN:formic acid (50:50:0.1). A Perkin Elmer Sciex API III triple quadrupole mass spectrometer was tuned and calibrated by a single pass through the French press at 6000 psi. Insoluble material was removed by two centrifugation steps at 20,000 × g for 10 min. The soluble extract was frozen and stored at −80 °C for future use. For amide digestion, purified cell walls were suspended in 30 ml of 50 mM Tris-HCl, pH 7.5. To this, 5 μg of purified Gst-Atl or 5 μl of His₆-CwlA containing E. coli extract was added and incubated at 37 °C.

Cell wall digests were cleared by two centrifugation steps at 32,500 × g for 15 min. The supernatant was loaded onto a column packed with 1 ml of Ni-NTA resin (Qiagen) equilibrated with 10 ml of buffer Y (10% glycerol, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) at a flow rate of 0.5 ml/min. After loading, the column was washed with 10 ml of buffer Y followed by 2 ml of 2% buffer Z (buffer Y supplemented with 500 mM imidazole) in buffer Y. Seb-MHL-Cws was eluted by a step gradient with 50% buffer Z in buffer Y. Absorbance was monitored at 280 nm, and peak fractions were analyzed for the presence of purified surface protein by 12% SDS-PAGE.

**Preparation of COOH-terminal Anchor Peptides—**Purified Seb-MH-Cws was precipitated by the addition of an equal volume of methanol: chloroform (5:1) and centrifuged for 10 min at 11,000 × g. The aqueous phase was discarded, 1 volume of methanol was added to the supernatant and vortexed, and protein was sedimentsed by centrifugation for 10 min at 11,000 × g. The supernatant was discarded, and the precipitate was washed with 1 volume of methanol. Pellets were dried under vacuum in a Speed-Vac concentrator (Savant), and protein was resuspended in 1–3 ml of 70% formic acid. About 100 μl of 20% sodium borohydride (33) was added, and the cleavage reaction was incubated for 16–18 h at room temperature in the dark. Samples were dried under vacuum and washed twice with 0.5 ml of water. Pellets were dissolved in 1–2 ml of buffer A (6 M guanidine hydrochloride, 0.1 mM NaHPO₄, 0.01 mM Tris-HCl, pH 8.0) and loaded onto a column packed with 1 ml of Ni-NTA Sepharose pre-equilibrated with 10 ml of buffer A. The column was washed with 10 ml of each buffer A, buffer B (8 M urea, 100 mM NaHPO₄, 0.01 mM Tris-HCl, pH 8.0), and buffer C (same as buffer B, but pH 6.3). Peptides were eluted with 2 ml of buffer D (6 M guanidine hydrochloride, 0.5 mM acetic acid, pH 4.3) and subjected to RP-HPLC on C18 column (2 × 250 mm, C18 Hypersil, Keystone Scientific). Separation was carried out at 40 °C with a flow rate of 0.2 ml/min and a linear gradient starting 10 min after injection from 99% H₂O, 0.1% trifluoroacetic acid to 60% H₂O, 0.1% trifluoroacetic acid, and 40% CH₃CN in 35 min followed by a steep gradient to 99% CH₃CN in 5 min. Eluate absorbance was monitored at 215 nm, and 1-min fractions were collected.
flow injection (10 μl/min) of a mixture of PPG 425, 1000, 2000 (3.3 × 10⁻³, 1 × 10⁻³, and 2 × 10⁻³ μl, respectively) in water:methanol (1:1) containing 2 mM ammonium formate and 0.1% CH₃CN. Calibration across the m/z range 10–2400 was achieved by multiple ion monitoring of eight PPG solution signals (singly charged ions at m/z 520.4). The ion spray voltage was operated at 4.5 kV using hydrocarbon-depleted air for spray nebulization, and spectra were generated with a curtain gas produced from the vapors of liquid nitrogen.

RESULTS

Solubilization of Surface Protein by Enzymatic Digestion of the Staphylococcal Cell Wall—Previous work developed an experimental scheme for the analysis of anchor peptides of staphylococcal surface proteins (18). The hybrid Seb-MH₆₆-Cws contains the protein A sorting signal fused to the COOH terminus of Seb. At the fusion joint, a methionine followed by six histidines is inserted (Fig. 1). After enzymatic solubilization of the staphylococcal cell wall, Seb-MH₆₆-Cws is purified by affinity chromatography on Ni-NTA Sepharose. CnBr cleavage at methionyl releases COOH-terminal anchor peptides that are isolated by another round of affinity chromatography and analyzed for their linked cell wall structures. B, diagram of the staphylococcal peptidoglycan illustrating the sites of enzymatic hydrolysis for muramidase (M), lysostaphin (L), amidad (A), and the staphylococcal phage φ11 hydrolyase (modified after Strominger and Ghuysen (4)). The glycan chains are composed of the repeating disaccharide N-acetylmuramic acid-(β1–4)-N-acetylgalactosamine (MN-GN).

FIG. 1. Enzymatic solubilization of Seb-MH₆₆-Cws from the staphylococcal peptidoglycan. A, schematic diagram of Seb-MH₆₆-Cws harboring the sorting signal (Cws) of protein A fused to the COOH terminus of Seb. The sorting signal contains an LPXTG motif followed by a hydrophobic domain (black box) and tail of mostly positively charged residues (boxed +). At the fusion site with Seb, a methionine followed by six histidines is inserted. After solubilization from the staphylococcal cell wall, Seb-MH₆₆-Cws is purified by affinity chromatography on Ni-NTA Sepharose. CnBr cleavage at methionyl releases COOH-terminal anchor peptides that are isolated by another round of affinity chromatography and analyzed for their linked cell wall structures. B, diagram of the staphylococcal peptidoglycan illustrating the sites of enzymatic hydrolysis for muramidase (M), lysostaphin (L), amidad (A), and the staphylococcal phage φ11 hydrolyase (modified after Strominger and Ghuysen (4)). The glycan chains are composed of the repeating disaccharide N-acetylmuramic acid-(β1–4)-N-acetylgalactosamine (MN-GN). C, Coomassie-stained 12% SDS-PAGE of Seb-MH₆₆-Cws solubilized from the peptidoglycan of S. aureus with lysostaphin (L), mutanolysin (M), φ11 hydrolyase (φ11), Gst-Atl amidase (A, Atl), or His₆-CwlA amidase (A, CwlA). About 1 μg of purified protein was loaded on each lane.

staphylococcal cell wall, Seb-MH₆₆-Cws is purified by affinity chromatography on nickel Sepharose. COOH-terminal anchor peptides are generated by CnBr cleavage at methionyl, purified by a second round of affinity chromatography, and analyzed by mass spectrometry.

Isolated staphylococcal peptidoglycan was digested with lysostaphin, φ11 hydrolase, mutanolysin, autolysin, or lysin A, and purified Seb-MH₆₆-Cws was analyzed on Coomassie-stained SDS-PAGE (Fig. 1C). As previously observed, lysostaphin-cut Seb-MH₆₆-Cws migrated faster on SDS-PAGE than the two species obtained after φ11 hydrolase digestion (18). Mutanolysin, an N-acetylmuramidase that cut at the β1–4 glycosidic linkage between MurNAc and GlcNAc (23), solubilized Seb-MH₆₆-Cws as a spectrum of fragments with increasing mass, all of which migrated more slowly on SDS-PAGE than the lysostaphin-digested counterpart. N-Acetylmuramyl-L-alanine amidases, S. aureus Atl (25), and B. subtilis CwlA (27) also released Seb-MH₆₆-Cws as a spectrum of fragments with different mass. Most amidase-released species migrated more slowly on SDS-PAGE than then lysostaphin-cut counterpart, indicating surface protein attachment to cross-linked peptidoglycan. These results suggested that φ11 hydrolase cut the staphylococcal cell wall not only at the N-acetylmuramyl-L-Ala amide bond but also at the peptidoglycan cross-bridge, thereby releasing Seb-MH₆₆-Cws linked to unsubstituted (non-cross-linked) tetrapeptide. Amidase-treated samples also contained a prominent species of Seb-MH₆₆-Cws that migrated faster on SDS-PAGE than the lysostaphin-solubilized counterpart. This species was characterized to be the product of proteolytic cleavage between the glutamine (Q) and the alanine (A) upstream of the

FIG. 2. Characterization of mutanolysin-solubilized anchor peptides. A, mutanolysin-solubilized Seb-MH₆₆-Cws was purified, precipitated with methanol/chloroform, and cleaved with CnBr. COOH-terminal anchor peptides were purified by affinity chromatography on nickel-Sepharose and subjected to RP-HPLC. B, MALDI-MS analysis of the peak eluting at 34% CH₃CN revealed a spectrum of signals. Inset, m/z 2700–2900 drawn to an expanded scale. Ion signals marked with an asterisk were generated by muramidase-solubilized anchor peptides that had lost some of their amino sugar residues.
LPX TG motif during sample preparation (AQ/ALPETG, data not shown).

Characterization of Muramidase-solubilized Anchor Peptides—To analyze the structure of mutanolysin-solubilized anchor peptides, the CnBr cut fragments were purified by affinity chromatography. The eluate was rechromatographed by RP-HPLC to separate COOH-terminal anchor peptides from contaminating peptides generated by incomplete CnBr cleavage (18). Two peaks of absorption at 215 nm were observed for these samples. The peak that eluted at 27% CH3CN was composed of COOH-terminal anchor peptides, whereas the peak that eluted at 33% CH3CN contained additional peptide sequence (NH2-VDSKDVKIEVYTNTKKGTMHHHHHHAQA-LPET-cell wall anchor) (Fig. 2A, data not shown). Mutanolysin-solubilized anchor peptides were analyzed by MALDI-MS (Fig. 2B). The mass of anchor peptide linked to a single murein pentapeptide unit, MurNAc-(L-Ala-D-iGln-L-Lys-(NH2-HHHHHHAQALPET-Gly5)-D-Ala-D-Ala-COOH)(b1–4)-GlcNA-(c1–4)-GlcNA, was calculated to be 2785.88 \([M+H]^{+}\), which was in agreement with the observation of a strong signal at \(m/z\) 2786.

Nevertheless, we also expected signals for anchor peptide linked to cell wall tetrapeptide as well as the \(N, O\)-6-diacetylated muramoyl species of the staphylococcal peptidoglycan (5). Close examination of the spectrum revealed compounds with \(m/z\) 2716.09, 2714.80, MurNAc-(L-Ala-D-iGln-L-Lys-(NH2-HHHHHHAQALPET-Gly5)-D-Ala-D-Ala-coOH)(b1–4)-GlcNA, whereas the observed ions at \(m/z\) 2757.53 and 2756.83 were explained as the \(N, O\)-6-diacetylated muramoyl species of the compounds with \(m/z\) 2785.88 and 2786.83.

### Table I

| Murein subunit | Wall peptide | 0 O-6-acetylation | 1 O-6-acetylation | 2 O-6-acetylation | 3 O-6-acetylation | 4 O-6-acetylation |
|---------------|--------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Monomer       | Tetrapeptide | 2716.09           | 2714.80           | 2757.53           | 2756.83           | 2785.88           |
| Monomer       | Pentapeptide | 2786.21           | 2785.88           | 2828.38           | 2827.91           | 2827.66           |
| Dimer         | Tetrapeptide | 3876.36           | 3918.77           | ND                | (3918.99)         | (3903.26)         |
| Dimer         | Pentapeptide | 3947.83           | 3990.70           | 4032.86           | 4032.10           | (4032.10)         |
| Trimer        | Tetrapeptide | 5039.45           | 5081.41           | 5121.07           | 5165.21           | 5165.21           |
| Trimer        | Pentapeptide | 5109.63           | 5153.98           | 5195.66           | ND                | (5195.66)         |
| Tetramer      | Tetrapeptide | ND                | 6243.41           | 6282.37           | 6328.83           | 6371.07           |
| Tetramer      | Pentapeptide | 6271.09           | 6312.85           | 6356.51           | 6396.27           | 6441.96           |

\(a\) Number of murein subunits (MurNAc-(L-Ala-D-iGln-L-Lys-(Gly5)-D-Ala)-(b1–4)-GlcNA) linked to surface protein.

\(b\) Status of the wall peptide within the terminal linked murein subunit, either pentapeptide (L-Ala-D-iGln-L-Lys-(Gly5)-D-Ala-D-Ala) or tetrapeptide (L-Ala-D-iGln-L-Lys-(Gly5)-D-Ala).

\(c\) Number of \(N\)-acetylmuramoyl acetylated at the O-6 position.

\(d\) Observed \(m/z\) and predicted average mass of compounds (in parentheses).

\(e\) ND, not detected.
Ions with \( m/z \) 2716, 2786, and 2856 differed by an average mass of 28 from those at \( m/z \) 2716, 2786, and 2828, respectively. Signals with mass deviation of 28 were observed routinely for CnBr-cleaved anchor peptides and were likely due to formylation of anchor peptides during the incubation in 70% formic acid (18).

Mutanolysin digestion of the staphylococcal cell wall released Seb-MH 6-Cws as a spectrum of fragments, indicating that anchor peptides must be tethered to cross-linked peptidoglycan. The mass of a murein subunit, MurNAc-(\(-\text{Ala} \text{-d-iGln-\( \text{L-Lys}\)-(Gly5)-D-Ala}\)-(β1–4)-GlcNAc), was calculated to be 1162.16 Da and 1204.19 Da for the \( N,\text{O}-6\)-diacetylated muramoyl species, respectively. Observed ion signals at \( m/z \) 2786, 3991, 5153, and 6357 differed from one another by mass increments of 1205, 1162, and 1204 Da, suggesting that these signals were generated by anchor peptides linked to one, two, or three murein subunits. \( N,\text{O}-6\)-diacetylation is known to occur at approximately half of all muramoyl residues in the staphylococcal peptidoglycan (5, 36). Thus, anchor peptides linked to two or three cell wall subunits carried at least one if not several diacetylated muramoyl. The predicted and observed \( m/z \) values of COOH-terminal anchor peptides linked to one, two, three, or four murein subunits each with varying degrees of acetylation at the muramoyl O-6 position were noted to be in

FIG. 5. MS/MS of the singly charged muropeptide ion at \( m/z \) 1068.7. A, the major compound contained within RP-HPLC peak A (Fig. 4) generated a singly charged ion at \( m/z \) 1068.7, which was subjected to CID in an MS/MS experiment. Fragment ion signals were compared with the structural model. B, proposed structure of the lysostaphin-solubilized compound at \( m/z \) 1068.7 based on the results of the MS/MS experiment: MurNAc-(\(-\text{Ala} \text{-d-iGln-\( \text{L-Lys}\)-(Gly3)-d-Ala}\)-(β1–4)-GlcNAc. See Table II for a complete listing of the observed daughter ions and their putative structural assignments.
Anchor Structure of Surface Proteins in S. aureus

Table II

Summary of daughter ions produced during MS/MS of the 1068.7 parent ion from HPLC peak A

| Observed m/z | Calculated m/z | Proposed structure |
|--------------|----------------|--------------------|
| 84           | 84.1           | Lys               |
| 137          | 138.1          | D-Ala-Gly         |
| 145          | 146.1          | GlcNAc            |
| 168          | 168.2          | GlcNAc            |
| 186          | 186.2          | GlcNAc            |
| 203          | 204.2          | GlcNAc            |
| 242          | 243.5          | L-Lys(Gly)        |
| 277          | 278.1          | MurNAc            |
| 314          | 314.4          | L-Lys(Gly)-d-Ala  |
| 326          | 327.4          | L-Ala-d-Gln-l-Lys |
| 330          | 332.4          | MurNAc(t-L-Ala)xH2O |
| 349          | 349.4          | MurNAc(t-Ala)d-l-Gly |
| 353          | 353.4          | d-Gln-t-Lys-(Gly)xH2O |
| 371          | 371.4          | d-Gln-t-Lys(Gly)  |
| 389          | 388.4          | L-Lys(Gly)-d-Ala-Gly |
| 425          | 424.5          | L-Ala-d-Gln-l-Lys(Gly)xH2O |
| 441          | 442.5          | L-Ala-d-Gln-l-Lys(Gly) |
| 458          | 459.5          | MurNAc(t-L-Ala)d-GlnxH2O |
| 476          | 477.5          | MurNAc(t-L-Ala)d-l-Gly |
| 499          | 498.5          | d-Gln-l-Lys(Gly)x-d-Ala-Gly-H2O |
| 517          | 516.6          | d-Gln-l-Lys(Gly)x-d-Ala-Gly |
| 556          | 555.6          | MurNAc(t-L-Ala)-o-Gln(t-L-Ala)xH2O |
| 573          | 573.6          | MurNAc(t-L-Ala)-o-Gln-t-Lys |
| 588          | 587.6          | L-Ala-t-Gln-l-Lys(Gly)x-d-Ala-Gly |
| 662          | 661.7          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)xH2O |
| 684          | 683.7          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)x2H2O |
| 702          | 701.8          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)xH2O |
| 719          | 719.8          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)xH2O |
| 750          | 749.8          | MurNAc(t-L-Ala)-o-Gln-l-Lys-d-Ala-Gly |
| 773          | 772.8          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)x-d-Ala |
| 790          | 789.8          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)x-d-Ala-Gly |
| 829          | 828.9          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)x2H2O |
| 847          | 846.9          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)x-d-Ala-Gly |
| 865          | 864.9          | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)x-d-Ala-Gly |
| 1068         | 1069.1         | MurNAc(t-L-Ala)-o-Gln-l-Lys(Gly)x-d-Ala-Gly-GlcNAc |

a See Fig. 5A for the relative intensity of daughter ions. Differences between observed and predicted m/z values are within tolerances of the experiment given the 1-dalton step size used during data acquisition and the degraded mass resolution used to enhance sensitivity.

Calculations are based on average masses according to the ChemDraw program.

d The proposed fragmentation scheme includes a complete series of ions of stepwise fragmentation after loss of the GlcNAc moiety.

Agreement (Table I), indicating that murein subunits linked to anchor peptides were either mono- or diacytated.

MALDI-MS signal intensity was found to decrease with the addition of each cell wall subunit to anchor peptide while the complexity of the spectrum simultaneously increased (see m/z 2786, 3991, 5153, and 6357). This can be explained by several observations. First, as detected on SDS-PAGE, less mutanolysin-released surface protein was linked to higher numbers of peptidoglycan subunits. Second, the addition of each cell wall subunit increased the number of possible mass combinations 2-fold while the mass of anchor peptide linked to unsubstituted tetrapeptide (NH2-HHHHHHAQALPET-Gly5)-D-Ala-COOH) (data not shown). Amidase removal of the MurNAc-GlcNAc disaccharide eliminated the degeneracy observed for mutanolysin-solubilized anchor peptides, thereby allowing detection of anchor peptides linked to as many as 11 murein subunits. For better analysis of the signals surrounding major ions, the mass spectrum was shown). Major signals of amidase-released anchor peptides were observed at m/z 2992, 3675, 4360, 5044, 5727, 6410, 7098, 7780, 8643, and 9149 (Fig. 3B). The difference in mass between these signals was 684, 683, 685, 684, 683, 688, 682, 683, and 686, respectively (mean 684.1). These results were in agreement with the mass of cross-linked cell wall tetrapeptide (NH2-t-L-Ala-d-iGln-l-Lys-(NH2-HHHHHHAQALPET-Gly5)-d-Ala-COOH) (data not shown).

Characterization of Amidase-solubilized Anchor Peptides—Gst-Atl solubilized Seb-MHP-Cws was cut with CrnBr and subjected to affinity chromatography on nickel Sepharose. Eluted compounds were chromatographed by RP-HPLC and COOH-terminal anchor peptides were detected in the peak that eluted at 23% CH3CN (Fig. 3A). When analyzed by MALDI-MS, this sample generated a spectrum of signals separated by regular mass increments (Fig. 3B). The mass of the anchor peptide linked to a single pentapeptide unit, NH2-t-L-Ala-d-iGln-l-Lys-(NH2-HHHHHHAQALPET-Gly5)-d-Ala-d-Ala-COOH, was calculated to be 2307.44 [M+H]+, which was in agreement with the observed signal at m/z 2308. A significantly weaker signal was detected at m/z 2235, an observation that was consistent with the mass of anchor peptide linked to unsubstituted tetrapeptide (calculated mass 2236.34, NH2-t-L-Ala-d-iGln-l-Lys-(NH2-HHHHHHAQALPET-Gly5)-d-Ala-COOH) (data not shown).
MH6-Cws was attached to either single murein pentapeptide and tetrapeptide subunits or to murein tetrapeptides that were cross-linked to the remainder of the staphylococcal peptidoglycan.

Characterization of Murein Subunits Linked to Anchor Peptides—If the multiple ion signals observed for the mutanolysin-solubilized anchor peptides were due to cross-linked peptidoglycan, lysostaphin cleavage of the pentaglycyl cross-bridge would be expected to generate murein subunits without linked anchor peptide. This was tested, and mutanolysin-released anchor peptides were first reduced by sodium borohydride treatment, desalted, and dissolved in Tris-HCl buffer for lyso- staphin treatment. Lysostaphin was removed from the digested sample by precipitation with 10% (v/v) trifluoroacetic acid, and the trifluoroacetic acid-soluble muropeptides were chromatographed by RP-HPLC using a gradient of phosphate-buffered methanol. Three major peaks of absorption at 215 nm (labeled A, B, and C) as well as several minor peaks were identified (Fig. 4). Fractions corresponding to peaks A, B, and C were desalted and analyzed by ESI-MS. Peak A generated a predominant ion at m/z 1068.7 as well as a less abundant ion at m/z 1125.7. These measurements were in agreement with the predicted mass of a singly charged reduced muropeptide (MurNAc-[L-Ala-D-iGln-L-Lys-(Gly5)-D-Ala]-COOH) with either three or four linked glycy1 residues (calculated masses of 1068.5 and 1125.5, respectively). ESI-MS analysis of peak B revealed a strong signal at m/z 1110.4 and a lesser ion at m/z 1167.4. These measurements differed from those observed in peak A by +41.9 daltons, indicating that peak B contained the N,O-6-diacylated counterparts of the compounds identified in peak A. Peak C contained ions at m/z 862.3 and 575.2, consistent with the calculated mass of the doubly and triply charged ions of lysostaphin-released anchor peptide (NH4+H H H H H H H H H A-QALPETGGG-COOH, 1722.81 Da).

To confirm the proposed structure of the murein subunit obtained by lysostaphin treatment of mutanolysin-released anchor peptides (peak A), the ion at m/z 1068.7 was subjected to CID in an MS/MS experiment. Analysis of the daughter ions generated by CID from the singly charged parent ion suggested the structure MurNAc-[L-Ala-d-iGln-L-Lys-(NH2-Gly-Gly)-D-Ala]-Gly-COOH (see Fig. 5 and Table II). Two other possible structures would generate an identical mass while harboring one or three glycy1 attached to the ε-amino of lysyl: MurNAc-[L-Ala-d-iGln-L-Lys-(NH2-Gly-Ala)-Gly-COOH](β1–4)-GlcNAc or MurNAc-[L-Ala-d-iGln-L-Lys-(NH2-Gly-Gly-Gly)-d-Ala-COOH](β1–4)-GlcNAc. However, the observed daughter ions at m/z 422, presumed structure NH2-L-Ala-d-iGln-L-Lys-(NH2-Gly-Gly)-COOH, and m/z 750, presumed structure MurNAc-L-Ala-d-iGln-L-Lys-d-Ala-Gly-COOH, suggested that two of three glycy1s were linked to the ε-amino of lysyl. This interpretation was in agreement with previous data regarding the preferred cleavage sites of the pentaglycyl cross-bridge by lysostaphin (37, 38). Taken together, the data revealed that mutanolysin-solubilized anchor peptides were attached mostly to cross-linked staphylococcal peptidoglycan.

DISCUSSION

We propose that surface proteins of S. aureus are linked to the cell wall via an amide bond between the carboxyl of thre- nine within the LPXTG motif and the amino of the pentaglycyl cross-bridge. Our hypothesis is based on the observation that surface proteins need enzymatic digestion of the bacterial cell wall to acquire solubility (39, 40). Digestion with mura- lytic enzymes cuts the peptidoglycan at specific sites and releases surface proteins linked to corresponding subunits of the staphylococcal cell wall. Lysostaphin cuts the glycy1-glycine bond of the pentaglycyl cross-bridge (41, 42) and solubilizes surface protein linked to either two or three glycine residues (17). Mutanolysin (N-acetylmuramidase) and Gs-Atl (N-acetylmu- ramyl-L-alanine amidase) release surface protein attached to a spectrum of peptidoglycan fragments composed of either cross-linked MurNAc-[L-Ala-d-iGln-L-Lys-(Glyx)-d-Ala](β1–4)-GlcNAc or NH2-L-Ala-d-iGln-L-Lys-(Glyx)-d-Ala, respectively. Finally, φ11 hydrolase solubilizes surface protein as two distinct species linked to unsubstituted L-Ala-d-iGln-L-Lys-(Glyx)-d-Ala-COOH or MurNAc-[L-Ala-d-iGln-L-Lys-(Glyx)-d-Ala-COOH](β1–4)-GlcNAc (18).

In our previous report, on the anchor structure of surface proteins of S. aureus we assumed that the φ11 enzyme displayed only amidase activity (19) and proposed that surface protein may be linked to free, unsubstituted cell wall subunits.
two activities that cut the cell wall at the N-acetylmuramyl-l-alanine and the D-alanyl-glycine bonds. We correct our model and propose that surface proteins are anchored to cross-linked peptidoglycan as well as cell wall pentaPeptidoglycan. Fig. 6 is a diagram of anchor peptide attached to a peptidoglycan dimer with the terminal subunit shown as acetylated murein-pentaPeptidoglycan. The calculated mass of this structure is 3989.06 Da, similar to a measurement obtained by MALDI-MS of mutanolysin-solubilized anchor peptides (Table I). Fig. 6 shows the muramoyl of the cell wall anchor structures to be diaacetylated; however, our data suggest that O-6-acetylation can occur at either, both, or neither of these subunits.

Although it appears that surface proteins are linked to murein subunits, the mechanism by which these polypeptides are incorporated into the peptidoglycan is still unclear. Future work will attempt to resolve whether surface proteins are attached to previously assembled peptidoglycan or linked to a precursor (43, 44), for example lipid II, which is subsequently incorporated into the cell wall via transglycosylation and transeptidation reactions (15). It is also conceivable that cell wall sorting occurs at sites of peptidoglycan assembly, assuming an abundance of free pentaglycyl cross-bridges at such locations. Isolation of sortase, the enzyme that catalyzes the anchoring of surface proteins, would permit the identification of such sites.

The cell wall sorting reaction has been proposed to be universal for Gram-positive bacteria (2). Evidence for this has recently accumulated, and cell wall sorting signals have been investigated in Listeria, streptococci, as well as other staphylococcal species (45–47). Both amidase-solubilized streptococcal M protein (48) and muramidase-solubilized internalin of Listeria (45) appear to migrate as species with cross-linked peptidoglycan on SDS-PAGE. Nevertheless, the universality of the sorting reaction in other Gram-positive bacteria requires more rigorous proof such as the purification and characterization of cell wall anchor structures from the peptidoglycan of these bacterial species.

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