Anchor Structure of Staphylococcal Surface Proteins

V. ANCHOR STRUCTURE OF THE SORTASE B SUBSTRATE IsdC

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Staphylococcus aureus sortase A cleaves surface protein precursors bearing C-terminal LPXTG motif sorting signals between the threonine and glycine residues. Using lipid II precursor as cosubstrate, sortase A catalyzes the amide linkage between the carboxyl group of threonine and the amino group of pentaglycine cross-bridges, thereby tethering C-terminal ends of surface proteins to the bacterial cell wall envelope. Staphylococcal sortase B also anchors its only known substrate, the IsdC precursor with a C-terminal NPQTN motif sorting signal, to the cell wall envelope. Herein, we determined the cell wall anchor structure of IsdC. The sorting signal of IsdC is cleaved between threonine and asparagine of the NPQTN motif, and the carboxyl group of threonine is amide-linked to the amino group of pentaglycine cross-bridges. In contrast to sortase A substrates, the anchor structure of IsdC displays shorter glycan strands and significantly less cell wall cross-linking. A model is proposed whereby sortases A and B recognize unique features of sorting signals and peptidoglycan substrates to deposit proteins with distinct topologies in the cell wall envelope.

The cell wall envelope of staphylococci and other Gram-positive bacteria functions as a surface organelle for microbial interaction with host tissues during infection. Many pathogenic strategies of staphylococci require the function of surface proteins that interact with extracellular matrices, specific host molecules, or target cells, thereby enabling bacterial adherence to tissues, target cell invasion, or evasion of immune responses (1–3). Many, but not all, surface proteins of staphylococci or other Gram-positive bacteria are anchored to the cell wall envelope by a mechanism requiring a C-terminal 35-amino acid sorting signal with an LPXTG motif (4–6). These surface proteins are synthesized as precursors with N-terminal signal sequences, thereby tethering C-terminal ends of surface proteins to the bacterial cell wall envelope. Herein, we determined the cell wall anchor structure of IsdC. The sorting signal of IsdC is cleaved between threonine and asparagine of the NPQTN motif, and the carboxyl group of threonine is amide-linked to the amino group of pentaglycine cross-bridges. In contrast to sortase A substrates, the anchor structure of IsdC displays shorter glycan strands and significantly less cell wall cross-linking. A model is proposed whereby sortases A and B recognize unique features of sorting signals and peptidoglycan substrates to deposit proteins with distinct topologies in the cell wall envelope.

The cell wall of Gram-positive bacteria is composed of peptidoglycan, a heteropolymeric macromolecule encompassing glycan strands and attached wall peptides (16, 17). Glycan strands, which consist of the repeating disaccharide MurNAc-(β1-4)GlcNAc (18), vary in length and contain up to 30 subunits, with a predominant length of 3–10 and an average of six disaccharide subunits (19). A short peptide component (L-Ala-d-iGln-(Gly5)-L-Lys-d-Ala) is attached via an amide bond between the lactyl moiety of MurNAc and the amino group of L-Ala (20–23). About 80–95% of the wall peptides of the assembled peptidoglycan are cross-linked, i.e. the amino groups of cross-bridges (pentaglycine (NH2-Gly5) in staphylococci) are amide-linked to the carbonyl groups of d-Ala within neighboring wall peptides (24–26).

During cell wall synthesis, soluble nucleotide-linked MurNAc pentapeptide precursor (UDP-MurNAc-L-Ala-d-iGln-L-Lys-d-Ala-d-Ala) is assembled in the cytoplasm and transferred to the bactoprenyl membrane carrier, thereby generating lipid I (C55-PP-MurNAc-L-Ala-d-iGln-L-Lys-d-Ala-d-Ala) (27–29). After disaccharide formation and addition of the pentaglycine cross-bridge, lipid II (C55-PP-MurNAc(L-Ala-d-iGln-(NH2-Gly5)-L-Lys-d-Ala-d-Ala)) (β1-4)GlcNAc), the biosynthetic substrate of extracellular cell wall assembly (30, 31), is translocated across the bacterial membrane (32). The peptidoglycan is finally polymerized via transglycosylation (33) and transpeptidation (34) reactions catalyzed by penicillin-binding proteins. Several lines of evidence indicate that lipid II functions as the cell wall substrate for sortase A, generating C55-PP-MurNAc(L-Ala-d-iGln-(surface protein-Gly5)-L-Lys-d-Ala-d-Ala)(β1-4)GlcNAc (11, 35–37). It is believed that subsequent peptidoglycan polymerization would result in the incorporation of the surface protein into the cell wall.

The Staphylococcus aureus isd locus is thought to be composed of three transcriptional units (isdA, isdB, and isdCDEF srtB isdG), each of which is regulated by Fur (38), a DNA-binding protein with affinity for canonical DNA sites (Fur boxes) (39). The isd locus is involved in bacterial heme iron

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¶ The abbreviations used are: MurNAc, N-acetylmuramic acid; d-iGln, d-isoglutamminyl; Iod, iron-regulated surface determinant; Fur, ferric uptake repressor; SBE, staphylococcal enterotoxin B; MHLs, Met-His2-CWS, cell wall sorting signal; Spa, staphylococcal protein A; Ni-NTA, nickel-nitritrolactacetic acid; PVDF, polyvinylidene difluoride; RP-HPLC, reversed-phase high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MALDI-TOF/TOF-MS, MALDI-TOF tandem mass spectrometry; CID, collision-induced dissociation.
uptake and specifies heme-binding proteins. IsdA, IsdB, and IsdC are cell wall-anchored proteins; IsdD, the IsdE lipoprotein, and the IsdF ATP-binding cassette transporter are membrane proteins; and IsdG is a cytoplasmic heme-cleaving enzyme (40, 41). IsdA and IsdB are sortase A (srtA)-anchored proteins with C-terminal LPXPTG motif sorting signals. They are displayed on the staphylococcal surface and are accessible to extracellular protease (40). In contrast, IsdC is a sortase B (srtB)-anchored protein with a C-terminal NPQKTG motif sorting signal. It is shielded from extracellular proteinase digestion by the cell wall envelope and is therefore not displayed on the staphylococcal surface (40).

Previous work showed that purified sortase B cleaves peptides bearing an NPQKTG motif in vitro, but left unresolved the IsdC cleavage site, IsdC anchor structure, and anchoring mechanism of sortase B (38). Here, we report the cell wall anchor structure of IsdC. In contrast to sortase A substrates, the anchor structure of IsdC displays shorter glycans strands and significantly less cell wall cross-linking. We discuss a model whereby sortases A and B recognize unique features of sorting signals and peptidoglycan substrates to deposit surface proteins with distinct topologies in the cell wall envelope.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—** S. aureus strains Newman (42), RN4220 (43), and SKM15 (RN4220 fur:cat) (38) have been described previously. To construct pAMP2 (encoding SEB-MH-ChWSSpa), DNA sequences specifying CWSSpa were amplified by PCR using S. aureus Newman chromosomal DNA as template and primers 6HisdCsig (5'-AAGGTGACATGCCATCACCATCACCATCACAAAGTAGAAAATCCAC-

**Fluorescently Labeled Monoclonal Antibody Preparation—** Antibodies used in this study were obtained from Novocea (Bohringer Mannheim). The antibodies used in the selective medium were ampicillin (100 μg/ml for E. coli) and chloramphenicol (10 μg/ml for S. aureus).

**Preparation of C-terminal Anchor Peptides—** Purified SEB-MH-ChWSSpa was mel Brooklyn, and SKM15 (pAMP2) were grown overnight at 37 °C. Cells were diluted 1:50 into fresh medium, grown to OD_{600} ~ 5.0, and incubated for 16 h at 37 °C with mild shaking. The cleaved sample was eluted by a step gradient of imidazole from 50 to 500 mM.

**MALDI Mass Spectrometry—** Dried HPLC fractions containing pep-
tides of interest were suspended in 15 μl of CH3CN/water/trifluoroacetic acid (30:70:0.1). MALDI mass spectra were obtained on a Reflectron time-of-flight instrument (ABI Biosystems) in the reflection mode. Samples were externally calibrated to a standard of 2,2'-dipyridyl. All samples were incubated at 37 °C. Cells were collected by centrifugation; supernatants were transferred to a PVDF membrane, and analyzed by staining with rabbit anti-IsdC polyclonal antisera and chemiluminescence staining.

RESULTS

Solubilization of IsdC from the Staphylococcal Cell Wall with Murine Hydrolases—IsdC is synthesized in the cytoplasm as a 227-amino acid precursor with an N-terminal signal peptide and a C-terminal NPQTN-type sorting signal (38). Using signal peptide algorithms and canonical sorting signal cleavage site predictions, Ala29 was identified as the N-terminal residue and Thr192 as the C-terminal residue of mature IsdC. The calculated molecular mass of predicted mature IsdC is 18,140 Da. To analyze the cell wall anchor structure of IsdC, we sought to solubilize the polypeptide from the staphylococcal peptidoglycan with murein hydrolases. Staphylococci pulse-labeled with [35S]methionine was used to release IsdC. Cell wall digestion with mutanolysin, a murein hydrolase that cuts the repeating disaccharide MurNAc-GlcNAc (49, 50), solubilized IsdC as a spectrum of seven fragments with different masses. The fastest migrating species displayed a mass similar to that of the 11 hydrolase-released IsdC, whereas the lysostaphin-released counterparts. Thus, it appears that IsdC is not the amide bond of MurNAc–L-Ala (45). Treatment with mutanolysin and lysostaphin did not release IsdC from the cell wall envelope (data not shown). Lysostaphin, a glycylglycine endopeptidase that cleaves pentaglycine cross-bridges of staphylococcal cell walls (Fig. 1A) (48), solubilized IsdC as a single species of 19 kDa (Fig. 1B). 11 hydrolase released the amide bond of MurNAc–L-Ala as well as the D-Ala–Gly peptide bond of the S. aureus peptidoglycan (45) and released IsdC as a species with a uni-
cell wall, as only treatment with lysostaphin and \( \Phi I I \) hydrolase, i.e., enzymes that directly cut cross-bridges or excise cross-bridges with wall peptides, released IsdC with a uniform mass. Cleavage of only glycan strands (mutanolysin) or cross-bridges (\( \Phi I I \Delta \)) each released distinct spectra of IsdC fragments, consistent with the hypothesis that the polypeptides must be linked to polymerized MurNac-GlcNac chains as well as wall peptides with some degree of cross-linking.

**Surface Display of SEB-MH6-CWSIsdC and SEB-MH6-CWSspa**—Fusion of sorting signal sequences to the C-terminal end of SEB, a highly expressed exoprotein, generates cell wall-anchored hybrids (14, 38). We wondered whether SEB hybrids anchored via NPQTN sorting signals display similar properties as wild-type IsdC. Previous work showed that IsdC is sequestered in the staphylococcal envelope and protected from extracellular proteases, whereas sortase A-anchored surface proteins (IsdA and IsdB) are not (40). To test whether SEB hybrids exhibit a similar phenotype, two plasmids encoding SEB-MH6-CWSspa and SEB-MH6-CWSIsdC were transformed into staphylococci. SEB-MH6-CWSspa represents a hybrid with a C-terminal LP\( \text{X} \)TG motif sorting signal (45), whereas SEB-MH6-CWSIsdC is anchored to the envelope via an NPQTN motif sorting signal (see below) (Fig. 2A).

Staphylococci were washed and suspended in suscrose buffer to stabilize bacterial protoplasts without cell walls that were generated in the course of this experiment. Staphylococcal suspensions were dispensed into three equal 1-ml aliquots. One of the two control samples was treated with proteinase K as a measure for protease sensitivity, whereas the other was left untreated. Proteins in all three samples were precipitated with 7.5\% trichloroacetic acid, washed with acetone, and separated by SDS-PAGE. After electrotransfer to a PVDF membrane, SEB hybrids were detected by staining with nickel-horseradish peroxidase conjugate.

Fig. 2B shows that sortase A-anchored hybrids (SEB-MH6-

**Fig. 3. Solubilization of SEB-MH6-CWSspa from staphylococcal cell walls.** A, plasmid pAMP2 expresses SEB-MH6-CWSspa from the \( \text{seb} \) promoter and sortase B (\( \text{sortA} \)) from the sortase A (\( \text{sortA} \)) promoter in \( \text{S. aureus} \). The MH6 sequence was inserted upstream of the NPQTN sorting signal and permits one-step purification of the recombinant polypeptide by affinity chromatography on Ni-NTA. Eluted peptides were cleaved with CNBr and repurified for mass spectrometric analysis of IsdC anchor peptides. B, Coomassie Blue-stained SDS-polyacrylamide gel of Ni-NTA-purified SEB-MH6-CWSIsdC, released by digestion of isolated staphylococcal murein sacculi with the muretic enzymes lysostaphin (L), mutanolysin (M), \( \Phi I I \) hydrolase (\( \Phi I I \)), and \( \Phi I I \Delta \) o-Ala-Gly endopeptidase (\( \Phi I I \Delta \)). P2 precursors and anchored species are indicated.

**Fig. 4. Mass spectrometric analysis of lysostaphin-released anchor peptides.** A, MALDI-TOF mass spectrum of SEB-MH6-CWSspa anchor peptides released from staphylococcal murein sacculi. The cell wall preparation was treated with lysostaphin, and the lysate was subjected to Ni-NTA affinity chromatography. Purified SEB-MH6-CWSspa was digested with CNBr to generate C-terminal anchor peptides, which were isolated by a second chromatography on Ni-NTA and then separated by RP-HPLC. Anchor peptides eluted at 14\% CH\textsubscript{3}CN and were subjected to MALDI-TOF-MS. B, proposed structures for the species detected by MALDI-TOF-MS in A. Obs., observed; Calc., calculated; C, MALDI-TOF/TOF mass spectrum of \( m/z \) 1809.02 identified in A. 

CWSspa\( ^{\text{SPA}} \) were completely digested by extracellular proteinase K treatment (lane 3), whereas sortase B-anchored SEB-MH6-CWSIsdC was not (lane 6). However, SEB-MH6-CWSIsdC is not intrinsically resistant to protease digestion, as treatment of lysostaphin-solubilized cell wall proteins with proteinase K degraded the polypeptide (lanes 2 and 5). Thus, SEB-MH6-

**Solubilization of SEB-MH6-CWSIsdC from the Staphylococcal Cell Wall with Murein Hydrolases**—Previous work reported that SEB-CWSIsdC, a hybrid generated by fusion of the C-
terminal end of enterotoxin B to the cell wall sorting signal of IsdC, is anchored to the staphylococcal cell wall (38, 40). To analyze the anchor structure of IsdC, we generated SEB-MH₆₆-CWSIsdC, a hybrid polypeptide with an engineered insertion of a Met-His₆ sequence upstream of the IsdC cell wall sorting signal. Our overall experimental strategy resembles that used for LPXTG anchor structure determination (14, 15), whereby cell wall-anchored SEB-MH₆₆-CWSIsdC can be solubilized with different murein hydrolases and then purified by affinity chromatography on Ni-NTA. Eluted proteins are cleaved at methionyl residues with CNBr (51), and C-terminal peptides are purified by a second round of affinity chromatography on Ni-NTA. Eluted NPQTN anchor peptides can then be subjected to mass spectrometric analysis. To achieve a high efficiency of anchoring of the SEB-MH₆₆-CWSIsdC chimera, we overexpressed it along with sortase B and thus generated pAMP2 (Fig. 3A). The plasmid was transformed into the mutant strain S. aureus SKM15 (38) to mimic iron starvation conditions.

To test whether SEB-MH₆₆-CWSIsdC is anchored to the staphylococcal peptidoglycan in a similar manner as full-length IsdC, the hybrids were solubilized with different murein hydrolases (Fig. 1B), (48), solubilized SEB-MH₆₆-CWSIsdC from isolated staphylococcal murein sacculi as two species that migrated at 28 and 30 kDa, respectively. The faster migrating species is composed of cell wall-anchored products (see below), whereas the slower migrating species represents the P2 precursor with an uncleaved sorting signal, which was also found in cell wall lysates generated with other murein hydrolases (Fig. 3B, arrows). The hypothesis of P2 accumulation was first proposed upon overexpression of SEB-CWSIsdC, as its P2 precursor absolutely requires sortase B to generate the faster migrating mature anchor species (38). This notion was corroborated here by mass spectrometric analysis of purified peptide fragments generated via CNBr cleavage of the purified SEB-MH₆₆-CWSIsdC P2 precursor, which revealed uncleaved sorting signal sequence (data not shown).

Φ11 hydrolase cleaves the peptidoglycan by hydrolyzing the MurNAc-α-L-Ala as well as ε-D-Ala–Gly amide bonds (Fig. 1A) (45). This enzyme released SEB-MH₆₆-CWSIsdC as a 28-kDa species (Fig. 3B). These observations suggest that the faster migrating SEB-MH₆₆-CWSIsdC species is indeed linked to the staphylococcal cell wall, as murein hydrolase treatment was required to release the polypeptide. Cell wall digestion with mutanolysin, a muramidase that cuts the repeating disaccharide MurNAc-GlcNAc (49, 50), solubilized SEB-MH₆₆-CWSIsdC as a spectrum of seven fragments with different masses. The fastest migrating species displayed a mass similar to that of Φ11 hydrolase-released IsdC, whereas the other six muramidase species migrated more slowly and with decreasing intensity upon SDS-PAGE. The Φ11Δ enzyme cleaves the peptide bond of ε-D-Ala–Gly, but not the amide bond of MurNAc-GlcNAc (49, 50), solubilized SEB-MH₆₆-CWSIsdC as a spectrum of seven fragments with different masses. Cleavage of only glycan strands or wall peptides each released distinct spectra of SEB-MH₆₆-CWSIsdC fragments, consistent with the hypothesis that the polypeptides must be linked to polymerized MurNAc-GlcNAc chains as well as wall peptides with a modest degree of cross-linking.

**Cell Wall Anchor Structure of Lysostaphin-Solubilized SEB-MH₆₆-CWSIsdC**—Isolated staphylococcal cell walls harboring anchored SEB-MH₆₆-CWSIsdC were treated with lysostaphin to cleave the bacterial peptidoglycan, and cell wall lysates were subjected to affinity chromatography on Ni-NTA. After elution with imidazole, SEB-MH₆₆-CWSIsdC was precipitated with chloroform/methanol, washed with methanol, and dried. Protein samples were suspended in formic acid, cleaved at methionyl residues with cyanogen bromide, washed, and dried prior to a second round of affinity purification. C-terminal anchor peptides were eluted from Ni-NTA with a declining pH gradient and subjected to RP-HPLC on a C₁₈ column. Anchor peptides that eluted at 14% CH₃CN and 0.01% trifluoroacetic acid were subjected to MALDI-TOF-MS, revealing a predominant ion signal at m/z 1809.02 (Fig. 4). This measurement is consistent with the structure of a C-terminal SEB-MH₆₆-CWSIsdC peptide anchor structure determined (14, 15), whereby muramidase treatment was required to release the polypeptide. Cell wall digestion with mutanolysin, a muramidase that cuts the repeating disaccharide MurNAc-GlcNAc (49, 50), solubilized SEB-MH₆₆-CWSIsdC as a spectrum of seven fragments with different masses. The fastest migrating species displayed a mass similar to that of Φ11 hydrolase-released IsdC, whereas the other six muramidase species migrated more slowly and with decreasing intensity upon SDS-PAGE. The Φ11Δ enzyme cleaves the peptide bond of ε-D-Ala–Gly, but not the amide bond of MurNAc-GlcNAc (49, 50), solubilized SEB-MH₆₆-CWSIsdC as a spectrum of seven fragments with different masses. Cleavage of only glycan strands or wall peptides each released distinct spectra of SEB-MH₆₆-CWSIsdC fragments, consistent with the hypothesis that the polypeptides must be linked to polymerized MurNAc-GlcNAc chains as well as wall peptides with a modest degree of cross-linking.

### Table 1

**Summary of daughter ions produced during tandem mass spectrometry of the lysostaphin-released parent ion at m/z 1809.02**

| Observed m/z   | Calculated m/z* | ∆obs-calc | Proposed structure | Ion type$^d$ |
|---------------|-----------------|-----------|--------------------|--------------|
| 110.07        | 110.07          | 0.00      | H                  | $^d$          |
| 275.16        | 275.13          | 0.03      | HH                 | $^b_2$       |
| 292.15        | 291.13          | 1.02      | T-Gly$_y$          | $^y_4$       |
| 412.23        | 412.18          | 0.05      | HHH                | $^b_3$       |
| 516.32        | 516.24          | 0.09      | PQT-Gly$_y$        | $^y_6$       |
| 549.29        | 549.24          | 0.05      | HHHH               | $^b_4$       |
| 686.36        | 686.30          | 0.06      | HHHHH              | $^b_5$       |
| 823.45        | 823.46          | -0.01     | HHHHHH             | $^b_6$       |
| 951.60        | 951.46          | 0.14      | HHHHHHK            | $^b_7$       |
| 1019.56       | 1019.49         | 0.07      | HHHHHHHK           | $^b_8$       |
| 1050.60       | 1050.52         | 0.08      | HKVENPQT-Gly$_y$   | $^{y_11}$    |
| 1123.64       | 1123.55         | 0.09      | HHHHHHKV           | $^b_9$       |
| 1179.65       | 1179.57         | 0.08      | HHHHHHKVE          | $^b_{10}$    |
| 1260.72       | 1260.61         | 0.11      | HHHKVENPQT-Gly$_y$ | $^{y_12}$    |
| 1293.74       | 1293.61         | 0.13      | HHHHHHKVEN         | $^{b_{10}}$  |
| 1397.78       | 1397.67         | 0.11      | HHHKVENPQT-Gly$_y$ | $^{y_12}$    |
| 1518.82       | 1518.72         | 0.10      | HHHHHKVENPQ        | $^{b_{12}}$  |
| 1534.85       | 1534.73         | 0.12      | HHHHHKVENPQT-Gly$_y$ | $^{y_{14}}$ |
| 1671.78       | 1671.79         | -0.01     | HHHHHKVENPQT-Gly$_y$ | $^{y_{15}}$ |
| 1734.81       | 1733.81         | 1.00      | HHHHHKVENPQT-Gly$_y$ | $^{b_{15}}$ |

$^a$ Calculations were based on average masses obtained with the MS-Product internet tool in ProteinProspector version 4.0.5 (available at prospector.ucsf.edu/ucsfhtml4.0/msprod.htm) (60).

$^b$ Values represent the difference between the observed and calculated masses of daughter ions.

$^c$ The nomenclature used refers to the NH$_2$- and CO$_2$H-terminal cleavage fragments according to Biemann (61).

$^d$ Immonium ion.

$^e$ Internal ion. The calculated mass corresponds to the sum of the residue masses (M + H$^+$).
that terminates at Thr\textsuperscript{192} and is amide-linked to three glycines (calculated \(m/z\) 1809.92). The second most abundant ion signal at \(m/z\) 1752.00 was interpreted as a C-terminal anchor peptide linked to two glycines (Fig. 4). To test whether \(m/z\) 1809.92 encompasses the predicted sequence H\textsubscript{6}KVENPQT-Gly\textsubscript{3}, the ion signal was subjected to collision-induced dissociation (CID), and daughter ion spectra were collected via MALDI-TOF/TOF-MS (Fig. 4C). The observed daughter ions are listed in Table I together with their predicted \(m/z\) values and CID fragmentation patterns, which confirmed the peptide sequence H\textsubscript{6}KVENPQT-Gly\textsubscript{3}. Several other ions in the spectrum of Fig. 4A were identified by MALDI-TOF/TOF-MS as N-terminal amino-formylated or amino-carbamylated anchor peptides carrying two or three C-terminal glycines (data not shown). Formylation and carbamylation of anchor peptides occur during CNBr cleavage (carried out in 70% formic acid) and affinity chromatography (performed with urea-containing buffers) of anchor peptides, respectively (15).

**Cell Wall Anchor Structure of \(\Phi 11\) Hydrolase-solubilized SEB-MH\textsubscript{6}-CWS\textsubscript{IsdC}**—Isolated staphylococcal cell walls harboring anchored SEB-MH\textsubscript{6}-CWS\textsubscript{IsdC} were digested with \(\Phi 11\) hydrolase, and SEB-MH\textsubscript{6}-CWS\textsubscript{IsdC} was purified from cell wall lysates as described above. After cleavage with CNBr, C-terminal peptides were repurified and subjected to RP-HPLC on a C\textsubscript{18} column. \(\Phi 11\) hydrolase-released anchor peptides eluted at 41% CH\textsubscript{3}CN and 0.01% trifluoroacetic acid and were subjected to MALDI-TOF-MS (Fig. 5A). Three clusters of ion signals were observed. The predominant ion signals in the first cluster, \(m/z\) 2321.17 and 2392.20, were explained as anchor peptides linked to cell wall tetrapeptides (NH\textsubscript{2}-L-Ala-D-iGln-L-Lys-(NH\textsubscript{2}-H\textsubscript{6}KVENPQT-Gly\textsubscript{3})-D-Ala-CO\textsubscript{2}H, calculated \(m/z\) 2321.48) and cell wall pentapeptides (NH\textsubscript{2}-L-Ala-D-iGln-L-Lys-(NH\textsubscript{2}-H\textsubscript{6}KVENPQT-Gly\textsubscript{3})-D-Ala-D-Ala-CO\textsubscript{2}H, calculated \(m/z\) 2321.20), respectively (Fig. 5B). The presence of cell wall pentapeptides indicates lack of cross-linking with other murein subunits. To test the predicted structure, the parent ion at \(m/z\) 2321.17 was subjected to CID, and daughter ion spectra were collected (Fig. 5C). Table II summarized the observed ions and their presumptive structures and fragmentation patterns, which are consistent with the structure of the branched peptide NH\textsubscript{2}-L-Ala-D-iGln-L-Lys-(NH\textsubscript{2}-H\textsubscript{6}KVENPQT-Gly\textsubscript{3})-D-Ala-CO\textsubscript{2}H. Other ions in the first \(\Phi 11\) hydrolase cluster, \(m/z\) 2349.16 and 2420.19, were explained as formylated species of anchor peptides and therefore suggest the absence of cross-linking to other murein subunits. The signals at \(m/z\) 3004.49, 3032.48, 3075.52, and 3103.52, which were isolated by a second chromatography on Ni-NTA and then separated by RP-HPLC, were subjected to MALDI-TOF-MS. Anchor peptides eluted at 41% CH\textsubscript{3}CN and were subjected to MALDI-TOF-MS. B, proposed structures and their calculated \(m/z\) values for the species detected by MALDI-TOF-MS in A. (\(d\)-Ala) indicates the presence of a cell wall pentapeptide and the absence of cross-linking with other murein subunits. C, MALDI-TOF/TOF mass spectrum of \(m/z\) 2321.17 identified in A.

SEB-MH\textsubscript{6}-CWS\textsubscript{IsdC} was digested with CNBr to generate C-terminal anchor peptides, which were isolated by a second chromatography on Ni-NTA and then separated by RP-HPLC. Anchor peptides eluted at 41% CH\textsubscript{3}CN and were subjected to MALDI-TOF-MS. B, proposed structures and their calculated \(m/z\) values for the species detected by MALDI-TOF-MS in A. (\(d\)-Ala) indicates the presence of a cell wall pentapeptide and the absence of cross-linking with other murein subunits. C, MALDI-TOF/TOF mass spectrum of \(m/z\) 2321.17 identified in A.
compound was subjected to CID in a MALDI-TOF/TOF-MS experiment. Consistent with a previous report using mass spectrometry to analyze the structure of peptidoglycan fragments (47), the predominant CID daughter ions resulted from the breakage of the (β1–4)-glycosidic bond of GlcNAc-MurNAc (observed m/z 2668.94, calculated m/z 2667.81) (Table III). However, many other, less abundant ions were detected and could be structurally assigned to N- or C-terminal CID fragments of the structure depicted in Fig. 6 (Table III).

In addition to the murein pentapeptide anchor structures, mass spectrometric experiments identified m/z 2801.40, the murein tetrapeptide anchor, as the second most abundant ion (GlcNAc(β1–4)MurNAc-(L-Ala-D-iGln-L-Lys-(NH-KVENPQT-Gly5))d-Ala, calculated m/z 2800.95) (Fig. 6A). MALDI-TOF/TOF-MS analysis of this ion corroborated this hypothesis (data not shown). As already reported for other anchor peptides, amino-formylated (m/z 2901.46) and amino-carbamylated (m/z 2915.43) forms of m/z 2872.44 were also detected (Fig. 6A).

The MALDI-TOF mass spectrum in Fig. 6A reveals a second cluster of muramidase-released anchor peptides with linked peptidoglycan. Three ion signals at m/z 4035.96, 4063.99, and 4078.02 were interpreted as C-terminal anchor peptides linked to the murein disaccharide tetrapeptide-disaccharide pentapeptide (calculated m/z 4034.21) and its amino-formylated (calculated m/z 4062.20) and amino-carbamylated (calculated m/z 4077.21) species, respectively. Although several attempts were made to identify Φ11 hydrolase- or muramidase-released anchor peptides with higher degrees of murein cross-linking, these experiments failed to reveal higher degrees of cross-linked peptidoglycan fragments tethered to IsdC anchor peptides. Furthermore, sortase B-anchored SEB-MH4-CWSisdC was linked to N,O-6-diacetylated murein subunits (data not shown), a modification that is known to occur at approximately half of all MurNAc residues in the staphylococcal peptidoglycan (25, 52). Thus, unlike sortase A-anchored surface proteins, the cell wall anchor structure of IsdC reveals predominantly short glycan strands and a non-cross-linked peptidoglycan.

**DISCUSSION**

Staphylococcal sortases anchor proteins to the bacterial cell wall envelope. Sortase A recognizes its substrates by the presence of an LPXTG motif, a site that is subsequently cleaved between the threonine and glycine residues (11). The enzyme performs a transpeptidation reaction whereby the

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**Table II**

| Observed m/z | Calculated m/z<sup>a</sup> | Δobs-calc<sup>b</sup> | Proposed structure | Ion type<sup>d</sup> |
|-------------|-----------------|-----------------|------------------|-----------------|
| 110.10      | 110.07          | 0.03            | H                |                 |
| 275.13      | 275.13          | 0.00            | HH               |                 |
| 412.21      | 412.18          | 0.00            | HHH              |                 |
| 549.27      | 549.24          | 0.00            | HHHH             |                 |
| 686.30      | 686.30          | 0.00            | HHHHH            |                 |
| 823.39      | 823.46          | 0.07            | HHHHHH           |                 |
| 951.47      | 951.46          | 0.01            | HHHHHHK          |                 |
| 1050.50     | 1050.52         | -0.02           | HHHHHHKV         |                 |
| 1179.54     | 1179.57         | -0.03           | HHHHHHKVE        |                 |
| 1290.63     | 1293.61         | 0.02            | HHHHHHKVEN       |                 |
| 1498.78     | 1498.75         | 0.02            | L-Ala-d-lGln-l-Lys-(NH-KVENPQT-Gly5)-d-Ala | y14 |
| 1637.65     | 1635.82         | 1.83            | L-Ala-d-lGln-l-Lys-(NH-HHKVENPQT-Gly5)-d-Ala | y15 |
| 1772.75     | 1772.88         | -0.13           | L-Ala-d-lGln-l-Lys-(NH-HHKVENPQT-Gly5)-d-Ala | y16 |
| 1909.93     | 1909.94         | -0.01           | L-Ala-d-lGln-l-Lys-(NH-HHKVENPQT-Gly5)-b-Ala | y17 |
| 2047.89     | 2047.90         | 0.19            | L-Ala-d-lGln-l-Lys-(NH-HHHHHKVENPQT-Gly5)-b-Ala | y18 |
| 2183.84     | 2184.06         | -0.22           | L-Ala-d-lGln-l-Lys-(NH-HHNNHKVENPQT-Gly5)-b-Ala | y19 |

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<sup>a</sup> Calculations were based on average masses obtained with the MS-Product internet tool in ProteinProspector version 4.0.5 (60).

<sup>b</sup> Values represent the difference between the observed and calculated masses of daughter ions.

<sup>c</sup> The nomenclature used refers to the N- and C-terminal cleavage fragments according to Biemann (61); an exception is indicated below. Ion number was calculated considering the histidine tail as the main N terminus.

<sup>d</sup> Immonium ion.
C-terminal threonine of the LPXTG-bearing polypeptide is amide-linked to pentaglycine cross-bridges (13–15). Several lines of evidence suggest that sortase A utilizes lipid II as a peptidoglycan substrate and that proteins linked to lipid II are incorporated into the growing cell wall envelope via transpeptidation and transglycosylation reactions (35, 36). Consistent with the view that sortase A and cell wall biosynthetic enzymes use the same substrates, anchored proteins are tethered to a highly cross-linked peptidoglycan, embedded in chains of glycan strands of five or more MurNAc-GlcNAc disaccharides in length and cross-linked to as many as 11 cell wall peptides (15). Sortase A-anchored polypeptides are displayed on the bacterial surface and evenly distributed within the staphylococcal envelope (53, 54). Because cell wall synthesis and sortase A anchoring may be constitutive and their cell wall anchor structure? Although we do not yet fully understand their cell wall anchor structure? Although we do not yet fully understand the molecular mechanisms that dictate the travels of proteins within the envelope, we believe that the cell wall substances of sorting reactions may determine the subsequent location of proteins in the envelope. For example, if sortase A used lipid II as a substrate in vivo, thereby ensuring uniform distribution of anchored proteins in the envelope, sortase B would then employ a different peptidoglycan substrate, probably not a biosynthetic precursor, but an assembled cell wall,
Anchor Structure of Sortase B Substrates

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