Characterization of *Staphylococcus aureus* Cell Wall Glycan Strands, Evidence for a New β-N-Acetylglicosaminidase Activity*

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Using sequential digestion with the glycol-glycine endopeptidase lysostaphin followed by the pneumococcal N-acetylmuramyl-l-alanine amidase (amidase), the glycan strands of the peptidoglycan of *Staphylococcus aureus* were purified and analyzed by a combination of reverse-phase-high pressure liquid chromatography (HPLC) and mass spectrometry. Reverse-phase-HPLC resolved the glycan strands to a family of major peaks, which represented oligosaccharides composed of repeating disaccharide units (N-acetylglucosaminyl-[β-1,4]-N-acetylmuramic acid) with different degrees of polymerization and terminating with N-acetylmuramic acid residues at the reducing ends. The method allowed separation of strands up to 23–26 disaccharide units with a predominant length between 3 and 10 and an average degree of polymerization of ~6. Glycan strands with a higher degree of polymerization (>26 disaccharide units) represented 10–15% of the total UV absorbing glycan material. A unique feature of the staphylococcal glycan strands was the presence of minor satellite peaks that were present throughout the HPLC elution profile eluting either just prior or shortly after the major oligosaccharide peaks. A number of observations including mass spectrometric analysis suggest that the satellites are the products of an N-acetylglicosaminidase activity that differs from the *all* gene product and that appears to be involved with modification of the glycan strand structure.

In contrast to the numerous high resolution studies on the structure and the mode of cross-linking of bacterial muropeptides, relatively little information is available on the structure of glycan strands, which, together with the peptide network, make up the backbone of bacterial cell walls. In fact, as of now, the only detailed information available concerning the structure and length distribution of cell wall glycan strands comes from *Escherichia coli* (1); only estimates of average length of glycan strands are available for *Staphylococcus aureus* cell walls (2).

The reasons why most studies on bacterial cell walls have concentrated on the structure and biosynthesis of the peptide network (3–6) with relatively little attention paid to the glycan component may be related to interest in the mode of action of β-lactam antibiotics, a widely used class of antimicrobial agents targeted on proteins (penicillin-binding proteins), that catalyze the transpeptidation of cell wall muropeptide components. No antimicrobial agent of comparable therapeutic importance is available for the transglycosylation reaction by which the disaccharide units (composed of N-acetyl-l-glucosamine-1,4-β linked to N-acetylmuramic acid) are linked together to form the glycan strands of the cell wall peptidoglycan. Yet, in our current view of cell wall biosynthesis, the first reaction by which disaccharide pentapeptide units are added to the preexisting peptidoglycan is transglycosylation catalyzed by two kinds of enzymes described so far: bifunctional class A penicillin-binding proteins, which, in addition to a penicillin-interacting transpeptidase domain at the C terminus, also possess an N-terminal transglycosylase domain (7, 8) and monofunctional glycosyltransferases (monofunctional enzymes insensitive to β-lactams that only catalyze the formation of β-1,4 glycosidic bonds (9, 10)). It may be anticipated that the appearance of the first *S. aureus* strains resistant to vancomycin therapy (11, 12) will stimulate studies on transglycosylases, because inhibition of cell wall synthesis by the glycopeptide class of antibiotics appears to occur at the transglycosylase level (13).

We used a combination of enzymatic hydrolysis, HPLC, and mass spectrometry to describe the structure and the length distribution of glycan strands of the *S. aureus* cell walls. Our observations suggest the presence in *S. aureus* of a hitherto unidentified N-acetylglicosaminidase activity that may be involved with processing the peptidoglycan at some stage of cell wall metabolism.

**EXPERIMENTAL PROCEDURES**

**Strains and Growth Conditions—** *S. aureus* strains listed in Table I were grown as described before (14). For labeling with N-acetyl-l-[3H]glucosamine ([1H]GlcNAc, 8.2 Ci/mmol) or l-[3,4,5-3H]lysine (98 Ci/mmol/Amersham Pharmacia Biotech), cells were grown on chemically defined medium (15) to which was added either [3H]GlcNAc (2 μCi/ml) or [3H]lysine (2 μCi/ml in chemically defined medium without l-lysine). **Peptidoglycan Purification—** Cell wall isolation and peptidoglycan purification was performed as described before (14). To minimize the chances of autolysis during harvesting and processing of cells for peptidoglycan preparation, cultures were rapidly chilled to 4 °C in an ice ethanol bath, centrifuged at 4 °C, and immediately added to an equal volume of boiling 8% SDS.

**Isolation and Purification of Glycan—** Glycan strands were isolated using a method described by Hara et al. (1), modified as follows. Purified peptidoglycan (5 mg/ml) was first hydrolyzed with recombinant lyso- staphin (1 μg/ml) (Sigma) in 25 mM potassium phosphate buffer, pH 7, 4°C.

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1 The abbreviations used are: HPLC, high pressure liquid chromatography; GlcNAc or G, N-acetyl-D-glucosamine; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; CHCA, α-cyano-4-hydroxycinnamic acid; ATT, 6-aza-2-thiodyamine; NC, nitrocellulose; 3-AQ, 3-aminoquinoline; PSD, post-source decay; MurNAc or M, N-acetylmuramic acid.
Characterization of Glycan Strands in the S. aureus Cell Wall

### Table I

| Strains | Relevant phenotype | Relevant genotype | Origin or reference | Average number of disaccharide repeating units of resolvable glycan | Percentage of long glycan strand material | Ratio of satellite peaks over major peaks |
|---------|-------------------|------------------|--------------------|---------------------------------------------------------------|----------------------------------------|------------------------------------------|
| COL     | Homogeneous McR   | meca<sup>a</sup> | RU collection      | 5.8 (0.5)                                                      | 8.8 (2.0)                              | 0.63 (0.08)                             |
| RUSAL9  | Mc<sup>b</sup>; Km<sup>b</sup>; Lyt | COL (at.: Tn 551); meca<sup>a</sup> | 23                 | 5.6 (0.1)                                                      | 7.7 (1.5)                              | 0.64 (0.04)                             |
| 27s     | Susceptible strain | meca<sup>a</sup>; NCTC8325 cured of phages φ11 and φ12 and lysogenized for phage 80a | Richard Novick    | 5.9 (0.4)                                                      | 15.1 (0.7)                             | 0.76 (0.05)                             |
| CTX50   | CTXR             | meca<sup>a</sup>; Laboratory step mutant of strain 27s | 19.20 | 4.6 (0.1)                                                      | 4.5 (0.7)                              | 1.46 (0.04)                             |

<sup>a</sup> HPLC analysis is capable of resolving glycan strands of up to 26 disaccharide units (up to retention times of 100 min, see Fig. 1). Average number of disaccharide repeating units was calculated as described by Harz et al. (1). Standard deviations are in parentheses based on at least three different peptidoglycan preparations for each strain.

<sup>b</sup> Composed of all glycan material with more than 26 disaccharide units (see Fig. 1).

The glycan strand pattern of strains 27s and COL were used as a reference for naming the satellite peaks and major peaks. Satellite peaks are all labeled with a number and a, b, or c in Fig. 1. Major peaks have only a number as label (see Fig. 1).

at 37 °C for 18 h with stirring. The solubilized S. aureus peptidoglycan was diluted with one volume of water. Murein stem peptides were cleaved from the peptidoglycan with purified pneumococcal amidasase (N-acetylmuramyl-l-alanine amidase, 50–100 μg/ml) by incubating samples for another 18 h at 37 °C with stirring. Samples were boiled for 3 min and microcentrifuged, and the pH of the supernatant was adjusted to 2 with phosphoric acid. Glycan strands were separated from stem peptides on a MonoS column (Amersham Pharmacia Biotech); adjusted to 2 with phosphoric acid. Glycan material was used over 90 min at a flow rate of 0.5 ml/min. Unresolved glycan peaks were only resolved as broad peaks although the pattern of peaks remained essentially the same as that of borohydride reduced samples (data not shown).2

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Methyl esters of glycan samples were prepared as follows. A solution of a 4-μl sample (about 50–100 pmol) in acetonitrile-water (9:1, v/v) was evaporated at 22 kV and a grid voltage of 73%. For each spectrum, 128 (or 256) laser shots were averaged. The ions of the α-cyano-4-hydroxy cinnamic acid (CHCA) dimer and Glu1-fibronectin B were used for external calibration.

Matrix solutions were prepared by dissolving 20 μg of CHCA or 6-aza-2-thiouridine (ATT), both available from Aldrich, in 1 μl of acetonitrile-water (9:1, v/v) followed by 200 μl of nitrocellulose (NC, Aldrich, 11.8–12.2%) in 1,2-dimethoxyethane. Methyl esters of glycan samples were prepared by a two-layer application technique: (i) 5–10 pmol in acetonitrile-water (9:1, v/v) was added to 0.5 ml of either CHCA/NC or ATT/NC solution on the sample plate. After 30 s, on the top of the dried gray layer was added 0.6 ml of nitrocellulose (NC, Aldrich, 5–10 pmol) in acetonitrile-water (9:1, v/v) was evaporated (Speed Vac) to dryness. To the dried residue, a solution of 30 μl of HCl (dry) in absolute methanol (prepared by dissolving 30 μl of acetyl chloride in 300 μl of absolute methanol) was added and introduced under a hood for 2 h. The mixture, after standing at room temperature for 2 h, was evaporated to dryness (Speed Vac) and reconstituted in 10 μl of acetonitrile-water (9:1, v/v).

A thin layer of matrix embedded in NC was prepared by depositing 0.5 μl of either CHCA/NC or ATT/NC solution on the sample plate. After 30 s, on the top of the dried gray layer was added 0.6 μl of sample (5–10 pmol) in acetonitrile-water (9:1, v/v). A cleanly defined spot with white crystals on the top of the matrix-NC layer was formed, which produced very strong and stable signals upon laser irradiation.

### Determination of the Reducing Ends in Glycan Fractions: Glycosylation and MALDI-PSD Analysis of Major Peaks 3 and 4—

The glycosylation reaction was carried as follows: 10 μl of each sample in 10% acetonitrile (around 10–20 pmol/μl) was added to 5 μl of 4% 3-aminoquinoline (3-AQ) in 80% methanol and 500 μl of glacial acetic acid. The solution was incubated at 70 °C for 30 min. Aliquots were directly taken for MALDI-MS analysis without additional treatment. Sample preparation was done by a two-layer application technique: (i) 0.5 μl of 1% ATT in 50% aqueous acetonitrile were deposited on the sample plate and allowed to dry, (ii) 2 μl of the reaction mixture was added to 1 μl of the ATT matrix solution and 0.5 μl of 0.5% aqueous fluoroacetic acid, (iii) 0.8 μl of the sample mixture was deposited on 0.5 μl of dried matrix. The fluoroacetic acid is used to promote the formation of protonated molecules. After drying, the light yellow mass was gently ground with the sealed end of a melting point capillary tube to induce the viscous mass to crystallize into finely divided crystals for analysis of MALDI-MS. Using the timed ion selector, the precursor ion [M-H]<sup>+</sup> at m/z 1579.6 (for peak 3) or [M-Na]<sup>+</sup> at m/z 2079.8 (for peak water at 0.5 ml/min. After washing for 1 min, the individual peaks of glycan material were eluted from the column with a single step of 25% acetonitrile in 0.5% trifluoroacetic acid. Each sample was lyophilized and analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Glycan fragments that were purified to determine the reducing end by derivatization were collected from HPLC separations of nonreduced glycan samples. Under these conditions, the glycan peaks were only resolved as broad peaks although the pattern of peaks remained essentially the same as that of borohydride reduced samples (data not shown). Major peaks 3 and 4 from strain COL were collected and desalted.

MALDI-MS—Mass spectra were obtained on a Voyager Elite MALDI-time-of-flight mass spectrometer (Perseptive Biosystems, Inc., Farmingham, MA), equipped with a pulsed nitrogen laser (337 nm, 3-ns pulse), using GRAMS software for data collection and analysis. Operating parameters included the accelerating voltage at 22 kV and a grid voltage of 73%. For each spectrum, 128 (or 256) laser shots were averaged. The ions of the α-cyano-4-hydroxy cinnamic acid (CHCA) dimer and Glu1-fibronectin B were used for external calibration.

Matrix solutions were prepared by dissolving 20 μg of CHCA or 6-aza-2-thiouridine (ATT), both available from Aldrich, in 1 μl of acetonitrile-water (9:1, v/v) followed by 200 μl of nitrocellulose (NC, Aldrich, 11.8–12.2%) in 1,2-dimethoxyethane. Matrix solutions were prepared by a two-layer application technique: (i) 5–10 pmol in acetonitrile-water (9:1, v/v) was evaporated (Speed Vac) to dryness. To the dried residue, a solution of 30 μl of HCl (dry) in absolute methanol (prepared by dissolving 30 μl of acetyl chloride in 300 μl of absolute methanol) was added and introduced under a hood for 2 h. The mixture, after standing at room temperature for 2 h, was evaporated to dryness (Speed Vac) and reconstituted in 10 μl of acetonitrile-water (9:1, v/v).

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4) was selected for MALDI PSD analysis. The acceleration voltage was 22 kV. Mass units were 61.5 of the calculated molecular masses.

RESULTS

Amidase Hydrolysis of the Peptidoglycan—To analyze quantitatively the glycan moiety of the S. aureus cell wall, it was essential to have complete cleavage of the stem peptides by an amidase. A candidate for this procedure was the pneumococcal amidase, because recent observations indicate that this enzyme can hydrolyze teichoic acid-free peptidoglycans (17). Preliminary tests indicated that whereas the pneumococcal amidase was inert to intact S. aureus cell walls (data not shown), it was able to hydrolyze [3H]GlcNAc-labeled peptidoglycan of S. aureus strains COL (methicillin-resistant) and 27s (methicillin-susceptible) although about 30% of the labeled material would still remain insoluble after three consecutive digestions. Because the S. aureus peptidoglycan has a much higher degree of cross-linking compared with the pneumococcal peptidoglycan (14, 18), it was conceivable that the incomplete hydrolysis was because of the secondary structure of S. aureus peptidoglycan, which blocked full access of the amidase to the peptidoglycan substrate. In an attempt to bypass this problem, peptidoglycan preparations were first digested with the endopeptidase lysostaphin to hydrolyze the oligoglycine bridges cross-linking the staphylococcal stem peptides. Sequential digestion of the S. aureus peptidoglycan with lysostaphin followed by the pneumococcal amidase allowed solubilization of >97% of

![HPLC profile of [3H]GlcNAc-labeled glycan strands from S. aureus strain COL.](image)

Purified glycan strands from strain COL labeled with [3H]GlcNAc were separated on wide pore Nucleosil C18 reverse-phase HPLC column using a 0–10.5% acetonitrile convex gradient in 100 mM sodium phosphate buffer, pH 2, for 90 min followed by a 30% acetonitrile step in the same buffer to elute the very long glycan strands. This method allowed separation of up to 23–26 major peaks (peaks labeled by numbers). Satellite peaks are numbered according to the closest major peak with a suffix a, b, or c. A common a, b, or c suffix does not necessarily represent similarity of structure, but simply refers to retention times related to that of the nearest major peak. Peaks labeled a always elute before the corresponding major peak, whereas peaks b and c elute after the corresponding major peak.

![Graph](image)
Purification of the Glycan Strands—At low pH (i.e., pH of 2) the N-acetyl-d-glucosamine and N-acetylmuramic acid components of the peptidoglycan are uncharged, whereas stem peptides are charged positively. Based on this principle, Harz et al. (1) used an anion exchange column (MonoS column with SO₃ as a functional group) to separate glycan strands from the stem peptides of E. coli. Before adapting this method as a preparative procedure for the separation of S. aureus glycan strands, it was necessary to exclude the possibility that the amide hydrolysis used in our procedure was incomplete in cleaving all N-acetylmuramyl-L-alanine bonds. Glycan fragments with attached stem peptides would remain positively charged, causing their loss to the stem peptide fraction, which is retained on the MonoS column.

Isolation of Peptide-free Glycan—S. aureus peptidoglycan from strains COL and 27s labeled with [3H]GlcNAc was doubly digested with lysostaphin and the amidase and separated with a MonoS column. 95% of the labeled material was eluted with the dead volume as expected for peptide-free glycan material. This was confirmed by the use of peptidoglycan labeled with L-[3H]lysine; after the double digestion, 99% of the L-[3H]lysine was retained in the MonoS column and eluted with the salt step. The 1% of labeled material unaccounted for would not interfere with the analysis of the glycan fraction.

The Nature of the 5% [3H]GlcNAc-labeled Material Retained on the MonoS Column—The 5% or less [3H]GlcNAc-labeled material retained on the anion exchange column was eluted with 1 M NaCl and analyzed by HPLC. This material eluted exclusively with the 30% acetonitrile step and was free of peptides as determined by [3H]lysine labeling. In another test, the 5% retained material was digested with mutanolysin to further confirm that no uncleaved muropeptides were present. Only disaccharides (GlcNAc-[β-1,4]-MurNAc), trisaccharides, tetrasaccharides, and free peptides were detected (data not shown), suggesting that this fraction of glycan strands was somehow physically trapped on the column by the stem peptides.

Analysis of the Peptide-free Glycan Strands by HPLC—The [3H]GlcNAc-labeled glycan material of the methicillin-resistant S. aureus strain COL was separated on a reverse-phase HPLC system detected by UV absorption and by flow scintillation analysis. The elution profiles of radioactive and UV absorbing peaks were identical. With the 30% acetonitrile step it was possible to quantitatively recover all glycan material injected into the HPLC system. The elution profile of staphylococcal glycan showed a “hedgehog” pattern composed of major peaks and minor satellite peaks that eluted either just before or shortly after the major glycan peaks from the HPLC column (Fig. 1) and that accompanied the major peaks throughout the entire elution profile. Resolution of peaks required prior reduction of the glycan material with sodium borohydride. Interestingly, the relative proportion of major peaks to the minor satellite peaks was reversed in the laboratory mutant CTX50 (19, 20) selected for resistance to cefotaxime, as compared with their representation in the parental strain 27s (Fig. 2).

The Structure of Major and Minor Glycan Components: Analysis of Muramidase Digests of the S. aureus Glycan by HPLC and MALDI-MS—To identify the structure of the major and minor components of the glycan spectrum, the complex HPLC elution pattern of the staphylococcal glycan was first simplified by in vitro treatment of the glycan preparation with a muramidase. Fig. 3 illustrates the HPLC analysis of glycan fragments generated from the glycan of strain COL after a short (2 h) as compared with prolonged (overnight) incubation with a muramidase in vitro. After a 2-h muramidase digestion, two major peaks (peak 1 and 2) were detectable with retention times of 11.6 and 22.0 min; two minor peaks were also present (peak 1b and 2a) with retention times of 13.4 and 20.7, respectively.

After overnight in vitro digestion with muramidase, peak 2 was drastically reduced with a parallel increase in peak 1, although the amounts of peaks 1b and 2a did not change, indicating that these fragments were poor substrates for the muramidase. The shift in the relative amounts of peak 2 to peak 1 after prolonged digestion with the muramidase and the HPLC retention times of these two components suggest that the structures of peaks 1 and 2 are GlcNAc-[β-1,4]-MurNAc and [GlcNAc-[β-1,4]-MurNAc]₂ respectively.

The four peaks obtained after overnight digestion of the glycan from strain COL with muramidase were also analyzed
were not affected by further incubation. Dase shifted a substantial amount of peak 2 to peak 1. Peaks 1b and 2a, and 2.

Extended incubation of the glycan strands with the muramidase for 2 h and through an overnight treatment as described under "Experimental Procedures," and the HPLC profile was compared. Four glycan strands of strain COL were digested with a muramidase. The glycan sample of strain COL showed that peaks 1, 2a, and 2 are glycan fragments.

Fig. 3. A, HPLC profiles of muramidase digests of the S. aureus glycan. The glycan sample of strain COL was digested with a muramidase for 2 h and through an overnight treatment as described under "Experimental Procedures," and the HPLC profile was compared. The proposed structures of peaks 1b and 2a suggest the activity of a glucosaminidase.

The Structure of Major and Minor Glycan Components: Analysis of the Entire Range of S. aureus Glycan Strands by MALDI-MS—Matrixes previously used for oligosaccharides have turned out to be unsuccessful for the analysis of glycan material. However, after derivatization to the corresponding methyl esters, peaks could be obtained with standard CHCA as the matrix. Still better results were obtained using a new matrix ATT embedded in nitrocellulose, which produced strong signals and unambiguous molecular masses for individual peaks. This method was used for obtaining the MALDI-MS spectrum of the complete range of glycan strands of strain COL. Fig. 4 shows a series of major peaks with molecular masses that fit the expected molecular mass of GlcNAc-(β-1,4)-MurNAc and its multimers. Consecutive peaks in the spectrum are separated by 492 Da, which represents the molecular mass of the methyl ester of a disaccharide repeat unit. MALDI-MS analysis allowed identification of glycan strands with a degree of polymerization of up to 12 disaccharide-repeating units (m/z 5945, Table III). The fact that glycan strands with more than 12 disaccharide units were not detected is possibly because of their lower abundance (see Fig. 1) or to their reduced MALDI-MS response.

Also shown in Fig. 4 are minor peaks, the molecular masses of which are consistent with the structures (GlcNAc-(β-1,4)-MurNAc)ₙ-GlcNAc or MurNAc-(GlcNAc-(β-1,4)-MurNAc)ₙ. These structures are consistent with a glucosaminidase activity as was already suggested by the analysis of muramidase hydrolysis products. (see Fig. 3, Table II, and above).

Analysis of purified individual peaks by MALDI-MS reinforced most of these conclusions (see Table III); purified major peaks had molecular masses consistent with multimers of GlcNAc-(β-1,4)-MurNAc. Therefore, the major peaks were numbered according to their apparent degrees of polymerization. Numbers assigned to satellite peaks in Fig. 1 and Table III refer to their retention times relative to the retention time of the nearest major peak. Satellite peaks marked with the letter a indicate that they elute before the corresponding major peak, whereas satellites marked with letters b and c refer to elution after a particular major peak.

The Structure and Nature of Reducing Terminals of the Major Glycan Components—The molecular mass of major peaks (Table III) does not allow one to distinguish between two alternative structures with identical molecular weights: (GlcNAc-(β-1,4)-MurNAc)ₙ or (MurNAc-(β-1,4)-GlcNAc)ₙ. These two structures only differ in the N-acetylmuramyl peptide of the reducing end of the glycan fragment. To distinguish between these two possibilities, we derivatized two purified major peaks (3 and 4) with 3-AQ and performed MALDI-PSD of the derivatives. Fig. 5 illustrates the fragmentation pattern obtained from peak 4, the tetramer of the disaccharide unit. The sodium adduct of the derivatized peak 4 was selected as precursor ([M + Na]⁺ at m/z 2079). The protonated molecule of derivatized peak 4 was also present ([M + H]⁺ at m/z 2057). PSD spectra of peak 4 contained predominantly fragments of the protonated molecule. The major fragment corresponds to the loss of a GlcNAc residue (see Fig. 5), which must have been released from the nonreducing end of the molecule. This observation suggests that the reducing end of the major peaks is carried by a MurNAc acid residue. Sequential loss of the next residue, a MurNAc residue, was inferred from the small peak (m/z 1578). This fragmentation pattern is consistent with previous data using 3-AQ derivatives. Because the charge is located on the basic side of the quinoline nitrogen of the 3-AQ group, the [M + H]⁺ ion of glycosylamine derivatives preferentially fragments from the nonreducing end allowing determination of the sequence of N-acetylmuramylamines residues in the polysaccharide (21, 22). Unexpectedly, after the initial loss of the GlcNAc residue further fragmentation appeared to proceed mainly from the reducing end. Loss of the 3-AQ moiety alone or with several N-acetylmuramylamine residues simultaneously with the GlcNAc residue at the nonreducing end was also detected (see Fig. 5). This unusual fragmentation seems to be associated with the presence of MurNAc acid residues as other neutral

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3 N. Xu, Z.-H. Huang, and D. A. Gage, submitted for publication.
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**Table II**

MALDI-MS analysis of the muramidase digestion products of the glycan strands after reduction

| Glycan peaks* from Fig. 4 | Reduced forms* | Positive Mode | Methyl ester derivatives* |
|--------------------------|----------------|---------------|-------------------------|
|                          | Negative Mode  | Positive Mode | Proposed structure      |
|                          | [M - H]        | [M + Na] - 2H | [M + Na]                 |
|                          | [M + Na]+      | [M + K]+      | Calculated M*           |
| 1                        | 498            | 520           |                         |
| 1b                       | 699            | n.d.*         |                         |
| 2a                       | 771            | 793           |                         |
| 2                        | 974            | 995           |                         |

* The glycan material was reduced with sodium borohydride.

**Fig. 4. Positive mode MALDI-MS analysis of the methyl ester derivatives of unfractionated glycan strands of the peptidoglycan of *S. aureus* strain COL.** The glycan strands were methyl-esterified and analyzed by MALDI-MS using a new matrix ATT (see "Experimental Procedures"). Glycan strands were detected as sodium adducts [M + Na]-. Molecular masses up to 5456 m/z were identified reflecting glycan strands with up to 11 disaccharide (GlcNAc-[(β-1,4)-MurNAc]) repeating units. Less abundant signals could also be identified that corresponded to losses of m/z 289 (minus a MurNAc methyl ester) or of m/z 203 (minus a GlcNAc).

oligosaccharides normally fragment exclusively from the non-reducing end (21, 22). Analysis of the 3-AQ derivative of the smaller glycan component (peak 3) gave results similar (data not shown) to the ones described for the 3-AQ derivative of peak 4, described above.

**The Structure and Nature of Reducing Termi of the Minor Satellite Components of Staphyloccocal Glycan—**The nature of some satellite peaks is unambiguous in light of our knowledge on peptidoglycan synthesis. Newly synthesized glycan strands are composed of repeating units of the disaccharide GlcNAc-[(β-1,4)-MurNAc]. The different degrees of polymerization of the GlcNAc-MurNAc unit correspond to the major peaks (see above). Removal of a GlcNAc from the reducing end of major peaks leaves a polysaccharide with an odd number of N-acetylhexosamines with a GlcNAc at the reducing termini (GlcNAc-[(β-1,4)-MurNAc]). The molecular masses of peaks 2b, 3 (only m/z 1720), 4a, and 5a (see Table III) can only be interpreted by such structures. Simultaneous removal of a GlcNAc from the nonreducing end and a MurNAc from the reducing end generates polysaccharides with an even number of N-acetylhexosamine with a GlcNAc residue at the reducing end (MurNAc-[(β-1,4)-GlcNAc]). Such fragments would have the same molecular mass as the major peaks, although one would expect them to elute differently from the major peaks. The molecular masses of peaks 3b (only m/z 1594), 4b, and 4c (only m/z 2579) can be interpreted by such structures.

The interpretation of the MALDI-MS spectra of the purified satellite peaks was complicated by the appearance of peaks 77 Da higher in mass, the origin and chemical nature of which we are not clear. Nevertheless, we believe that the structures proposed for the satellite components of the glycan (see Table III) are correct. Both types of glycan fragments (MurNAc-GlcNAc-[(β-1,4)-MurNAc]) and (MurNAc-[(β-1,4)-GlcNAc]) would be expected to be found based on the total muramidase hydrolysis of the glycan strands (see Fig. 3 and Table II). Only these two structures could generate the trisaccharide MurNAc-GlcNAc-MurNAc identified by muramidase hydrolysis of the glycan strands. Additionally, the structure of the major peaks and glycan strands with GlcNAc residues at both the nonreducing and reducing end are also unambiguous. The peaks 77 Da higher in mass did not appear in the muramidase digests, suggesting that they may be artifacts of the methyl esterification process. At the present time, we have no proposed structure for the +77 adducts.

**Average Length of the Glycan Strands Strain to Strain Variation—**The method used to analyze the glycan strand pattern in *S. aureus* strains COL and 27s allowed the separation of glycan strands up to a degree of polymerization of approximately 26 disaccharide units with an average of 6 units (Table I). Using UV absorption data, the HPLC resolvable material was estimated to represent 85–90% of the total glycan fraction. However, UV estimates may include errors, because the molar extinction coefficients are unknown and the baseline of HPLC elution profiles was not linear. Glycan strands labeled with [3H]GlcNAc were used in an attempt to confirm the UV data or obtain more accurate estimates. In strain COL, 90.8% of the total radioactivity could be resolved as individual peaks with 9.2% of the radioactive material corresponding to unresolvable long glycan strands eluting with the 90% acetonitrile step. Because of the similarity of results, we have continued to use UV absorption to estimate average length of the glycan strands, the percentage of the unresolved glycan material, and the ratio of satellite peaks to major peaks in different strains of *S. aureus*. Table I illustrates the results. *S. aureus* strains 27s and COL appear to have the same average of around six repeating disaccharide units in the fraction of resolvable glycan strands. On the other hand, strain 27s has a higher percentage of very long glycan strands (15.1% of its glycan material with a

### Table II

| Glycan peaks* from Fig. 4 | Reduced forms* | Positive Mode | Methyl ester derivatives* |
|--------------------------|----------------|---------------|-------------------------|
|                          | Negative Mode  | Positive Mode | Proposed structure      |
|                          | [M - H]        | [M + Na] - 2H | [M + Na]                 |
|                          | [M + Na]+      | [M + K]+      | Calculated M*           |
| 1                        | 498            | 520           |                         |
| 1b                       | 699            | n.d.*         |                         |
| 2a                       | 771            | 793           |                         |
| 2                        | 974            | 995           |                         |

* The glycan material was reduced with sodium borohydride.

**Taking into account the molecular masses of H, Na, and K (m/z 1, 23, and 39, respectively) all the mass spectrometry signals are consistent with the calculated molecular masses.

**Methyl esterification of the glycan material adds a methyl group at the lactyl moiety of each MurNAc residue, increasing the mass by 14 Da/MurNAc present. Taking into account the increment of 14 Da/MurNAc, all mass spectrometry signals are consistent with the calculated molecular masses within experimental error.

* Calculated molecular masses of the reduced forms.

* n.d., not detected.
Characterization of Glycan Strands in the S. aureus Cell Wall

The peptidoglycan of the bacterial cell wall is the product of the catalytic activity of two kinds of synthetic enzymes: the transglycosylases, which catalyze the incorporation of the disaccharide pentapeptide cell wall precursors into the preexisting peptidoglycan through the formation of 1,4-β-glycosidic bonds, and the transpeptidases, which catalyze the formation of peptide cross-links between the muropeptide units incorporated into the cell wall. Detailed information about the degree of peptide cross-linking in the S. aureus cell wall was obtained after digestion of purified peptidoglycan with a muramidase followed by separation of the different classes of muropeptide monomers and oligomers by HPLC (14). However, no comparable information has been available about the length distribution of glycan strands in S. aureus. The only information available in the literature is from the E. coli cell wall in which the glycans are composed of repeating units of the disaccharide GlcNAc-(β-1,4)-MurNAc with an 1,6 anhydro-N-acetylmuramic acid end, possibly generated by the in vivo activity of lytic transglycosylases (24).

**DISCUSSION**

The peptidoglycan of the S. aureus cell wall has a degree of polymerization higher than 26 as compared with 8.8% in strain COL. The average length of the glycan strands estimated in this study is lower than that estimated by Ward (2). Strain 27s also has a slightly higher ratio of satellite over major peaks compared with strain COL (0.76 and 0.63, respectively). An additional kind of strain-specific variation in the ratio of major to satellite glycan components was already noted in the case of the cefotaxime-resistant mutant CTX50 (see above).

**Impact of the Inactivation of the atl Gene on the Glycan Structure—**The structure of the satellite peaks identified in the glycan of S. aureus suggests that they may be the products of a glucosaminidase activity. For this reason, the glycan strands of the parental strain COL and its transductant derivative carrying a transposon-inactivated *atl* were compared (23). Fig. 6 shows that the two glycan profiles were identical. Because the Atl products are the major autolysins in S. aureus and the inactivation of the *atl* gene abolishes autolysis (23), it is unlikely that the satellite peaks resulted from autolysis or even partial autolysis.

**TABLE III**

MALDI-MS analysis of methyl ester derivatives of individual glycan peaks purified from strains COL and CTX50

| Glycan peak | [M + Na]$^+$ | Proposed structure | Calculated [M + Na]$^+$ |
|-------------|--------------|--------------------|------------------------|
| 2           | 1026         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 1025                   |
| 2b          | 1228         | (GlcNAc-(β-1,4)-MurNAc)$_b$-GlcNAc | 1228                   |
| 3           | 1517 and 1720| (GlcNAc-(β-1,4)-MurNAc)$_b$ and (GlcNAc-(β-1,4)-MurNAc)$_b$-GlcNAc | 1517                   |
| 3b          | 1390 and 1594| MurNAc-(GlcNAc-(β-1,4)-MurNAc)$_b$ and (MurNAc-(β-1,4)-GlcNAc)$_b$ | 1517                   |
| 4a          | 2212         | (GlcNAc-(β-1,4)-MurNAc)$_b$-GlcNAc | 2212                   |
| 4           | 2009         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 2009                   |
| 4b          | 2087         | (MurNAc-(β-1,4)-GlcNAc)$_b$ | 2009                   |
| 4c          | 1884         | MurNAc-(GlcNAc-(β-1,4)-MurNAc)$_b$ | 1806                   |
| 5a          | 2704         | (GlcNAc-(β-1,4)-MurNAc)$_b$-GlcNAc | 2704                   |
| 5           | 2502 and 2579| (GlcNAc-(β-1,4)-MurNAc)$_b$ and (MurNAc-(β-1,4)-GlcNAc)$_b$ | 2501                   |
| 5b          | 2374         | MurNAc-(GlcNAc-(β-1,4)-MurNAc)$_b$ | 2298                   |
| 6           | 2995         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 2993                   |
| 7           | 3487         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 3485                   |
| 8           | 3979         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 3977                   |
| 9           | 4471         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 4469                   |
| 10          | 4965         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 4961                   |
| 11          | 5456         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 5453                   |
| 12          | 5945         | (GlcNAc-(β-1,4)-MurNAc)$_b$ | 5945                   |

$a$ See Fig. 1 for corresponding peaks nomenclature.
$b$ Observed as $+77$ adduct.
In the studies described here, we undertook a detailed analysis of *S. aureus* glycan using HPLC and MALDI-MS. The majority of components separated from the glycans of *S. aureus* strains COL and 27s contained between 3 and 10 disaccharide units with the average represented by 6 disaccharides and with 10–15% of the glycan strands represented by strands of longer than 26 disaccharides. An earlier effort using cell walls instead of purified peptidoglycan and a method of less resolving power (labeling of the reducing ends of glycan chains with borohydride) resulted in somewhat different figures for the average length of glycan strands (2). These differences may be related to the different *S. aureus* strains used in the two studies.

The HPLC elution pattern of staphylococcal glycan was reminiscent of the pattern described for the *E. coli* glycan (1), which was shown to consist of repeating units of the disaccharide GlcNAc-(β1,4)-MurNAc with the predominant lengths of the glycan strands between 5 and 10 disaccharide units carrying a nonreducing 1,6-anhydro-N-acetyl muramic acid as an end group (1). However, the two glycans differed in important respects: (i) the *S. aureus* glycan had to be reduced with sodium borohydride to improve HPLC resolution, indicating the absence of anhydro N-acetylhexosamine end groups, which is a prominent feature of the *E. coli* glycan; and (ii) a unique feature of the staphylococcal glycan elution pattern, not seen with *E. coli*, was the consistent presence of minor satellite peaks.

The results of HPLC and MALDI-MS analysis indicate that the major peaks in the hedgehog pattern of the *S. aureus* glycan are composed of repeating units of the disaccharide GlcNAc-(β1,4)-MurNAc with reducing termini in the MurNAc residues. The molecular masses of the satellite peaks are consistent with the structures (GlcNAc-[β1,4]-MurNAc)_n, GlcNAc, MurNAc-(GlcNAc-[β1,4]-MurNAc)_n, or (MurNAc-[β1,4]-GlcNAc)_n. We suggest that the satellite peaks are the products of a glucosaminidase activity that modify the staphylococcal glycan structure in vivo.

Before accepting this proposal for the origin of the satellite components several alternative possibilities had to be considered. Because the glycan strands have a reducible end, strands that terminate with MurNAc residues are prone to β-elimination under alkaline conditions (25), which would increase the complexity of the HPLC profiles. However, such artifacts are unlikely to contribute to the satellite peaks, because under the conditions used in our experiments exposure to pH 9 was in the presence of sodium borohydride and for only 15–30 min. Under these conditions, reduction of the C1 of the MurNAc is preferred over β-elimination (26). Additionally, we did not identify by MS any glycan fragments consistent with β-elimination. Once reduced by sodium borohydride, the glycan strands are no longer prone to β-elimination.

A second conceivable artifact is that the contamination of the enzymes used for the glycan preparation with glycan hydrolase activities was excluded by the control experiments (see “Experimental Procedures”). The extra precautions used during wall preparation and the results of the experiment with the atl mutant make it also unlikely that the satellite components are products of an autolytic glucosaminidase activity.

In our current model of murein biosynthesis, the cell wall precursor GlcNAc-(β1,4)-MurNAc-pentapeptides incorporate into the preexisting peptidoglycan by the transglycosylase reaction. If no further modification occurs, the glycan strands are composed of repeating disaccharide units, and HPLC analysis of such glycan strands should resemble that seen in *E. coli* (1). An endogenous muramidase activity would cleave the MurNAc-(β1,4)-GlcNAc bond shortening the glycan strands by disaccharide units, which, however, would always be composed of even numbered N-acetylhexosamine components and would always carry the reducing group at the MurNAc termini. In contrast, a hypothetical endogenous glucosaminidase activity would hydrolyze the GlcNAc-(β1,4)-MurNAc bond creating glycan strands with an odd number of saccharides and glycans with a poly-MurNAc-[β1,4]-GlcNAc-polysaccharides sequence, thus complicating the HPLC elution profiles. Our observations suggest that the unique satellite peaks identified in the staphylococcal glycan represent products of such an endogenous glucosaminidase activity.

This hypothetical enzyme is most likely to have an endo- rather than exo-β-N-acetyl glucosaminidase activity. An endo type of enzyme cleaves glycosidic bonds along the polysaccharide backbone, whereas an exo type cleaves only from one end of the polysaccharide molecule. An exo type of enzyme would remove from a newly synthesized glycan strand (GlcNAc-[β1,4]-MurNAc), either the MurNAc from the reducing end or the GlcNAc from the nonreducing end. In such a scenario, glycan components would include newly synthesized glycan strands...
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(\text{GlcNAc-}[\beta-1,4]-\text{MurNAc})_n$, and processed glycan strands of one kind only ((GlcNAc-\text{[\beta-1,4]-MurNAc})_n)-(\text{GlcNAc or MurNAc-}[\text{GlcNAc-}[\beta-1,4]-\text{MurNAc})_n). Muramidase digestion of such glycan components ((GlcNAc-\text{[\beta-1,4]-MurNAc})_n)-(\text{GlcNAc or MurNAc-}[\text{GlcNAc-}[\beta-1,4]-\text{MurNAc})_n) would yield only one of the following trisaccharides: (GlcNAc-\text{[\beta-1,4]-MurNAc})-(\text{GlcNAc or MurNAc-}[\text{GlcNAc-}[\beta-1,4]-\text{MurNAc}), respectively, depending on from which end of the glycan strand the exo-enzyme digested. The appearance of both trisaccharides as shown by our data favors cleavage along the polysaccharide backbone by an endo type glucosaminidase. We cannot exclude a more complex scenario that would involve two distinct exo-glucosaminidases each one with a preference either for the nonreducing end or for the reducing end of the glycan strands, respectively.

The \textit{in vitro} enzymatic activity of this hypothetical glucosaminidase remains to be demonstrated. Earlier work has identified a bifunctional enzyme in \textit{S. aureus}, Atl, which has both glucosaminidase as well as amidase activity (27). However, our data with the \textit{S. aureus} strain in which the gene \textit{atl}, the genetic determinant of the bifunctional murein hydrolase, was inactivated indicate that Atl is not the enzyme involved with the generation of satellite components in the \textit{S. aureus} glycan. The reversal in the relative proportion of major peaks to satellite peaks in the glycan of the cefotaxime-resistant mutant CTX50 suggests an increased activity of the hypothetical glucosaminidase in this mutant. The overall length distribution of the \textit{S. aureus} glycan strands length may be the result of a dynamic balance between synthetic transglycosylases and glycan strand-degrading enzymes. Whether or not the hypothetical glucosaminidase contributes to such a processing of glycan chains in \textit{S. aureus} remains to be determined.

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