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# Peptidoglycan Synthesis and Structure in *Staphylococcus haemolyticus* Expressing Increasing Levels of Resistance to Glycopeptide Antibiotics

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**The structures of cytoplasmic peptidoglycan precursor and mature peptidoglycan of an isogenic series of *Staphylococcus haemolyticus* strains expressing increasing levels of resistance to the glycopeptide antibiotics teicoplanin and vancomycin (MICs, 8 to 32 and 4 to 16 μg/ml, respectively) were determined. High-performance liquid chromatography, mass spectrometry, amino acid analysis, digestion by R39 D,D-carboxypeptidase, and N-terminal amino acid sequencing were utilized. UDP-muramyl-tetrapeptide-D-lactate constituted 1.7% of total cytoplasmic peptidoglycan precursors in the most resistant strain. It is not clear if this amount of depsipeptide precursor can account for the levels of resistance achieved by this strain. Detailed structural analysis of mature peptidoglycan, examined for the first time for this species, revealed that the peptidoglycan of these strains, like that of other staphylococci, is highly cross-linked and is composed of a lysine muropeptide acceptor containing a substitution at its ε-amino position of a glycine-containing cross bridge to the D-Ala 4 of the donor, with disaccharide-pentapeptide frequently serving as an acceptor for transpeptidation. The predominant cross bridges were found to be COOH-Gly-Gly-Ser-Gly-Gly-$NH_2$ and COOH-Ala-Gly-Ser-Gly-Gly-$NH_2$. Liquid chromatography-mass spectrometry analysis of the peptidoglycan of resistant strains revealed polymeric muropeptides bearing cross bridges containing an additional serine in place of glycine (probable structures, COOH-Gly-Ser-Ser-Gly-Gly-$NH_2$ and COOH-Ala-Gly-Ser-Ser-Gly-$NH_2$). Muropeptides bearing an additional serine in their cross bridges are estimated to account for 13.6% of peptidoglycan analyzed from resistant strains of *S. haemolyticus*. A soluble glycopeptide target (L-Ala-γ-D-iso-glutamyl-L-Lys-D-Ala-D-Ala) was able to more effectively compete for vancomycin when assayed in the presence of resistant cells than when assayed in the presence of susceptible cells, suggesting that some of the resistance was directed towards the cooperativity of glycopeptide binding to its target. These results are consistent with a hypothesis that alterations at the level of the cross bridge might interfere with the binding of glycopeptide dimers and therefore with the cooperative binding of the antibiotic to its target in situ. Glycopeptide resistance in *S. haemolyticus* may be multifactorial.**

The glycopeptide antibiotics, including vancomycin and teicoplanin, are thought to act by specifically binding the terminal D-alanyl-D-alanine of the disaccharide-pentapeptide precursor as it appears at the surface of the cytoplasmic membrane (9). A propensity for dimerization leads to cooperative binding of the target, which increases the activity of the antibiotic (3, 13). An exception to this is teicoplanin, which apparently relies on an acyl membrane anchor to provide binding stability (3). High-level acquired resistance to glycopeptides (teicoplanin and vancomycin) has recently appeared in species of the genus *Enterococcus*, particularly in *Enterococcus faecalis* and *Enterococcus faecium* (2). The mechanism has been well delineated and is due to the synthesis of a novel cytoplasmic peptidoglycan precursor (4), UDP-muramyl-tetrapeptide-D-lactate, which has a markedly decreased affinity for the glycopeptide antibiotics vancomycin and teicoplanin (5). One of the worrisome possibilities is that this resistance, encoded within a transposon (2), frequently on plasmids, could undergo intergeneric transfer to a more virulent organism, such as *Staphylococcus aureus*. In fact, such transfer has been shown to occur in the laboratory (17).

For staphylococci, another pathway to resistance apparently exists via mutation (12, 18, 21–24). In contrast to the enterococci, staphylococci are generally more susceptible to vancomycin than to teicoplanin (12, 18, 23), the resistant isolates that have been tested and reported thus far are always more resistant to teicoplanin than to vancomycin (12, 15, 18, 21–24), and resistant derivatives are more readily selected with teicoplanin (12, 23). *Staphylococcus haemolyticus* strains resistant to teicoplanin were recognized before the release of the antibiotic to the market (18). The emergence of *S. haemolyticus* strains that are resistant to vancomycin during therapy in humans has also been reported (21).

The resistant strains of staphylococci express increased levels of a membrane protein variously described as either 35 or 39 kDa (6, 18, 24). Milewski et al. (16) have recently characterized this protein in *S. aureus* as a member of the family of dehydrogenases which includes the D-hydroxy acid dehydrogenase (VanH) associated with vancomycin resistance in enterococci (2). These data suggest that the mechanism of resistance

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TABLE 1. *S. haemolyticus* strains

| Strain | MIC (μg/ml) | |
|---|---|---|
| | Teicoplanin | Vancomycin |
| 18 | 8 | 4 |
| 18-73 | 16 | 8 |
| 18-88 | 32 | 16 |

in staphylococci may resemble that in enterococci. The *vanZ* gene of the vancomycin-resistant enterococci, by an as-yet-unknown mechanism, appears to code for a phenotype of teicoplanin resistance when it is expressed on a high-copy-number plasmid. This phenotype is reminiscent of glycopeptide resistance in staphylococci (1).

We herein describe the structures of cytoplasmic peptidoglycan precursor and of a significant portion of mature peptidoglycan of an isogenic series of *S. haemolyticus* strains expressing increasing levels of resistance to the glycopeptide antibiotics teicoplanin and vancomycin. Although the general structure of the peptidoglycan of some coagulase-negative species of staphylococci has been previously described (20), that of *S. haemolyticus* has not, to our knowledge, been examined prior to this work.

## MATERIALS AND METHODS

**Strains and growth conditions.** All strains used in this study are listed in Table 1. *S. haemolyticus* 18 (11) has been described previously. Strains 18-73 and 18-88 are consecutive one-step mutants of strain 18 with increasing levels of resistance to vancomycin. They were routinely cultured on brain heart infusion medium (Difco, Detroit, Mich.) at 37°C with agitation. The MICs of vancomycin and teicoplanin were determined by diluting the antibiotics twofold serially through brain heart infusion agar. Strains were spotted on the agar plates with a Steers replicator so that each spot contained $10^4$ CFU.

**Preparation of peptidoglycan precursors.** Peptidoglycan precursors were extracted and purified as previously described (4). In order to accumulate cytoplasmic precursors, cells were exposed for the last 30 to 60 min of growth to ramoplanin (either 0.5 or 1 μg/ml; Marion Merrell Dow, Lepetit Research Center, Geranzano, Italy), which prevents the formation of lipid intermediate II from I (25), or to vancomycin (1 mg/ml; Lilly, Paris, France). Digestion of the entire cytoplasmic extract with R39 D,D-carboxypeptidase was performed as previously described (4).

**Preparation of peptidoglycan.** Peptidoglycan was extracted essentially as described by de Jonge et al. (7). One difference was that the final pellet of purified peptidoglycan, usually between 15 and 30 mg, was digested in its entirety for 16 h at 37°C in 25 mM phosphate buffer (pH 6.5) containing 10 mM $MgCl_2$ with egg-white lysozyme (200 μg/ml) and M-1 muramidase (250 μg/ml) (Sigma Chemical Corp., St. Louis, Mo.). The enzymes were then inactivated by boiling for 3 min in water. After centrifugation the supernatants were stored at −20°C. We estimate that 85 to 95% of staphylococcal peptidoglycan was digested by this treatment (8).

**Separation of muropeptides.** Before separation the sample was mixed with an equal volume of 0.5 M borate buffer (pH 9) and reduced with sodium borohydride for 15 min at room temperature. The pH of this solution was adjusted to 2 with orthophosphoric acid. Samples were kept at −20°C. The high-performance liquid chromatography (HPLC) system consisted of an L6200 A pump and an L4250 UV detector (Merck), a 717 autosampler (Waters Corporation, Milford, Mass.), and a D2500 Chromatointegrator (Merck).

Samples were applied to a 3-μm Hypersil $C_{18}$ reversed-phase column (250 by 4.6 mm; Interchim) guarded by a Lichrospher 100 RP18 (5 μm; Merck) precolumn. The column was eluted at a flow rate of 0.5 ml/min with a linear gradient of 0.05% trifluoroacetic acid (TFA) in water to 20% $CH_3CN$ with 0.035% TFA in 90 min, starting 10 min after injection of the sample. The elution was continued for 20 min with 0.035% TFA in 20% $CH_3CN$. The column temperature was maintained at 30°C, and the eluted compounds were detected by $A_{210}$.

**Identification of the peaks.** Liquid chromatography-mass spectrometry (LC-MS) was performed with a Hewlett-Packard HP1090 Series II liquid chromatograph coupled to either a Finnigan (San Jose, Calif.) TSQ700 triple-quadrupole mass spectrometer or a Sciex (Thornhill, Ontario, Canada) API-III triple-quadrupole mass spectrometer, both equipped with nebulizer-assisted electrospray sources. Data acquisition was performed between 500 and 1,900 Da with scan times on the order of 2 to 3 s. The fact that molecules of higher mass were multiply charged allowed identification of these structures. No eluent splitting was used with the Finnigan instrument, whereas the eluent flow entering the Sciex was split 10:1 so that about 20 μl/min was entering the mass spectrometer. MS-MS was performed on singly and doubly charged protonated molecules with either helium or argon as the collision gas. To determine the relative contribution of each mass species to a given peak, when appropriate, the mass spectra were deconvoluted to convert all multiply charged ions into singly charged species. The resulting peak heights were then considered to be proportional to the abundance of each component.

Confirmations of the muropeptide structures deduced from the mass spectrometry were accomplished with different samples either by fragmentation with an MS-MS system or chemically after purification of the peak by HPLC with the gradient described above during a 60-min run and a 5-μm Lichrospher 100 RP18 column (125 by 4 mm; Merck). The *N*-acetyl-muramicitol concentration and amino acid composition of the muropeptides were determined after hydrolysis by 6 N HCl at 95°C for 16 h by the Waters AccQ Tag method. The different components were separated by HPLC and detected with a Waters 474 scanning fluorescence detector and a column (150 by 3.9 mm) supplied with the Waters kit; the gradient recommended by the manufacturer was used. The sequence of amino acids within cross bridges was determined by Edman degradation with material from purified muropeptides as described above.

**Competition of soluble glycopeptide target for vancomycin.** Staphylococcal strains were grown on agar plates at 37°C overnight. Cells were collected with an inoculating loop and suspended in Mueller-Hinton broth (Difco) adjusted to pH 6.8 with 1 N HCl to an optical density at 260 nm of 0.5. The suspension was diluted 1,500-fold, and 60 ml of the mixture was layered on 75-ml Mueller-Hinton (pH 6.8) agar plates and allowed to settle several minutes before the excess broth was removed. The plates were allowed to dry. A fixed concentration of vancomycin (20 μg/25 μl of reaction mixture) was incubated briefly with increasing concentrations of L-Ala-γ-D-iso-glutamyl-L-Lys-D-Ala-D-Ala (Sigma Chemical Corp.) before the entire mixture was loaded on sterile 7-mm-diameter paper disks that had been placed on the seeded agar plates. The plates were incubated at 37°C overnight, and the zone sizes were measured. A standard curve of increasing concentrations of vancomycin was similarly prepared and read as a control.

## RESULTS

**Cytoplasmic precursors of peptidoglycan biosynthesis.** Soluble UDP-containing precursors were extracted from strains 18 and 18-88 as previously described (4). The extractions were performed with accumulation of cytoplasmic precursors in the presence of ramoplanin. The results are shown in Fig. 1. Peak 1 represents UDP-muramyl-pentapeptide, with a monoisotopic mass of 1,149 Da as confirmed by amino acid analysis. Peak 2 contains UDP-muramyl-tetrapeptide-D-lactate, with a monoisotopic mass of 1,150 Da. Aside from its mass, the material in peak 2 runs in the same position as control UDP-muramyl-tetrapeptide-D-lactate purified from vancomycin-resistant enterococci. No mass could be detected in this area for precursors extracted from strain 18, as shown in Fig. 1. To further characterize the material in peak 2 of strain 18-88, the extracts of strains 18 and 18-88 were subjected to digestion by purified R39 D,D-carboxypeptidase (4). This enzyme cleaves the terminal D-alanine or D-lactate. The digested extracts were subjected to LC-MS (data not shown); in both cases, the material in the pentapeptide peak had disappeared while a new peak, with a retention time of 13 min, appeared with a monoisotopic mass of 1,078 Da, consistent with a tetrapeptide. The R39 carboxypeptidase-treated extract from strain 18-88 showed no detectable peak at 27 min, suggesting that the material in the undigested sample running with this retention time and having a mass of 1,150 Da was also converted to UDP-muramyl-tetrapeptide and therefore was UDP-muramyl-tetrapeptide-D-lactate. Integration of the areas of the various peaks (data not shown) reveals that peak 2 accounted for 1.7% of total identifiable UDP-muramyl precursors in strain 18-88 and less than 0.2% in strain 18.

**Peptidoglycan structure of *S. haemolyticus* strains with increasing resistance to glycopeptides.** The separations by HPLC of muropeptides derived from the peptidoglycan of the susceptible *S. haemolyticus* strain 18 and its resistant derivatives, 18-73 and 18-88, are shown in Fig. 2. Each peak for which we were able to determine at least one molecular mass was as-

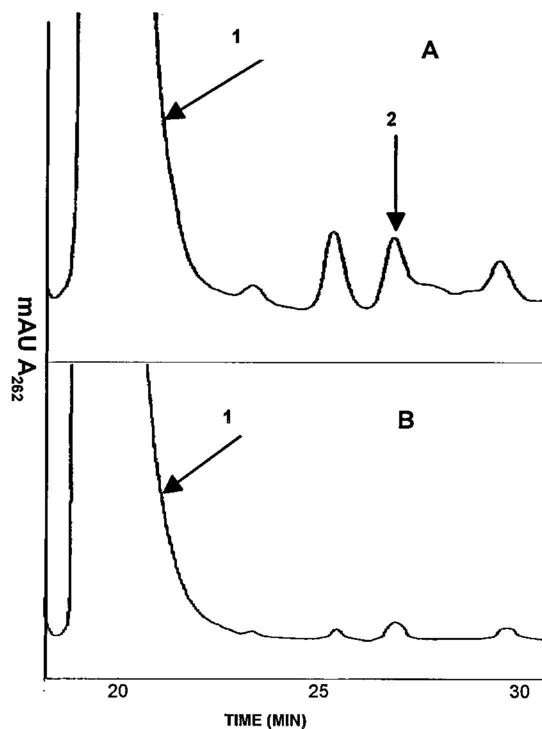



FIG. 1. Portion of the HPLC separation of UDP-muramyl precursors of cytoplasmic extracts of *S. haemolyticus* 18-88 (A) and 18 (B) as observed during LC-MS analysis. Peak 1 contains UDP-muramyl-pentapeptide with a monoisotopic mass of 1,149 Da, while peak 2 contains putative UDP-muramyl-tetrapeptide-D-lactate with a monoisotopic mass of 1,150 Da. No other mass signals were detected in this portion of the chromatogram. AU, absorbancy units.

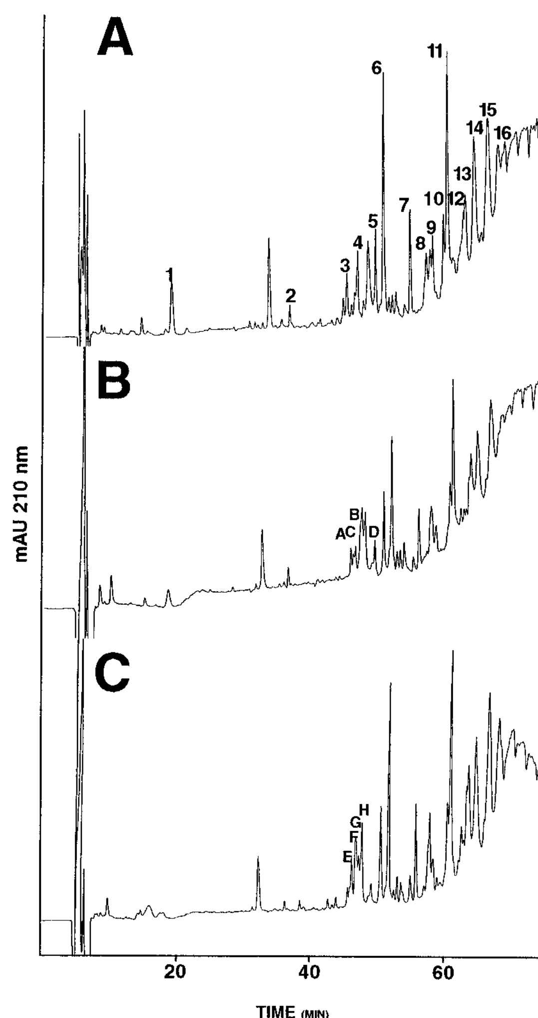



FIG. 2. HPLC separation of muropeptide fragments of *S. haemolyticus* 18 (A), 18-73 (B), and 18-88 (C). For strain 18, peaks containing components associated with at least one mass signal were given numeric designations. Not all peaks are labeled, since not all were associated with mass signals. For the chromatogram of strain 18-73, new peaks containing components with masses not seen in the peptidoglycan of strain 18 have been assigned letter designations. The chromatogram of strain 18-88 was compared with that for 18-73. AU, absorbancy units.

signed a number. The results of integration of peaks of typical chromatograms are shown in Table 2. As can be seen, the peptidoglycan of these strains, like that of other staphylococci, appears to be highly cross-linked (89% of peptidoglycan in dimers or higher-order polymers) (Fig. 2 and Table 2). Two pieces of evidence confirm that the material in peak 16 and beyond contains primarily muropeptides: amino acid analysis of this material confirms that there is little contamination, and our mass data on these peaks are consistent with the possibility that peak 16 contains pentameric muropeptides and other peaks, in some cases, contain higher-order polymeric muropeptides.

In the case of *S. haemolyticus*, multiple peaks (e.g., peaks 4, 11, 13, 14, and 15) contain two or more muropeptides as determined by mass spectrometry. When this was the case, the proposed muropeptide structures were given subdesignations (e.g., 4a, 4b, etc.), which are shown in Fig. 3. Amino acid analysis was performed for muropeptides 2, 6, and 7 as indicated in Table 2, and in each case the proposed structure was confirmed. The proposed sequence of amino acids in the cross bridge was based on N-terminal amino acid sequencing of purified material derived from peaks 6 and 7.

Muropeptides 1 to 4 could be the result of peptidoglycan breakdown. The structure contained in peak 1, with a mass of 498 Da, is presumed to represent the disaccharide GlcNAc-MurNAc and could be the result of an amidase activity. Muropeptide 2, which was purified and subjected to amino acid analysis (Table 2), contains no *N*-acetylglucosamine and could result from the activity of a glucosaminidase. Muropeptide 3 could result from endopeptidase activity similar to that of lysostaphin (19) and a carboxypeptidase. Muropeptide 4 could result from endopeptidase activity.

Structures 6 and 7 are monomeric disaccharide-pentapeptides with their cross bridges attached. Their structures were confirmed by analysis of amino acid content, and the positions of the alanine and serine in the cross bridge were assigned on the basis of amino acid sequence data from analysis by Edman degradation. The deduced structures are generally similar to those published previously for *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* (20).

Peaks 8, 9, 11, and 13 contain dimeric muropeptides for which the pentapeptide monomer has served as the acceptor molecule. Peaks 10 and 12 appear to contain dimeric muropeptides of the tripeptide-tripeptide variety. Peaks 14 and 15 contain trimeric muropeptides, again including a pentapeptide as an acceptor. Peak 16 (structure not shown) is a pentameric muropeptide. Mass data obtained from some later peaks (not shown) indicate that these too contain muropeptides.

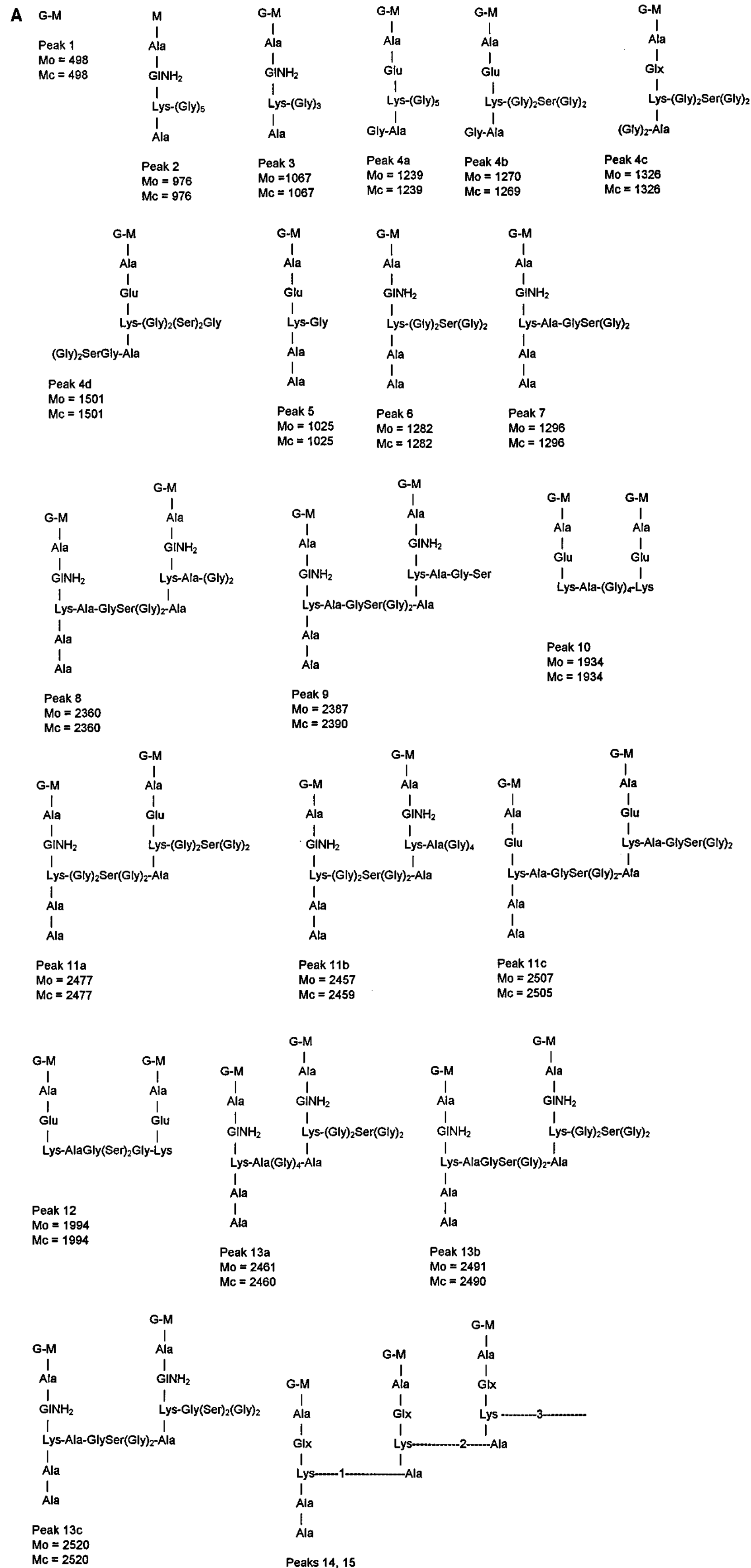
TABLE 2. Muropeptide composition of *S. haemolyticus* strains

| Peak number | % of total peptidoglycan[a] in strain: | | | Muramicitol[b,c] | Amino acid content[c] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 18 | 18-73 | 18-88 | | Glu | Lys | Ala | Gly | Ser |
| 1 | 1.0 | 0.9 | ND | | | | | | |
| 2 | 0.3 | 0.4 | ND | 0.7 | 0.5 | 0.7 | 2.5 | 4.5 | 0 |
| A | ND | 0.7 | ND | | | | | | |
| 3 | 0.8 | 2.4 | 1.8 | | | | | | |
| 4 | 1.1 | 1.3 | 3.8 | | | | | | |
| D | ND | 1.3 | ND | | | | | | |
| E | ND | ND | 0.9 | | | | | | |
| G | ND | ND | 0.8 | | | | | | |
| 5 | 1.1 | 2.5 | 1.8 | | | | | | |
| 6 | 3.1 | 5.1 | 3.8 | 1.2 | 0.9 | 0.9 | 3.1 | 3.8 | 0.9 |
| 7 | 1.3 | 1.8 | 1.4 | 0.7 | 0.9 | 0.8 | 4.2 | 3.4 | 0.7 |
| 8 | 1.3 | 3.1 | 2.0 | | | | | | |
| 9 | 1.3 | 1.3 | 0.8 | | | | | | |
| 10 | 1.3 | 2.5 | 1.8 | | | | | | |
| 11 | 3.9 | 5.6 | 4.8 | | | | | | |
| 12 | 2.3 | 0.9 | 1.5 | | | | | | |
| 13 | 2.0 | 2.9 | 3.0 | | | | | | |
| 14 | 4.9 | 5.1 | 6.3 | | | | | | |
| 15 | 6.3 | 5.3 | 8.0 | | | | | | |
| >15 | 66 | 56 | 60 | | | | | | |

[a] For strain 18-73, muropeptides 3, B, and C were contained within peak 3. For strain 18-88, muropeptides 3 and F were contained within peak 3, and muropeptides 4 and H were contained within peak 4. ND, not detected.
[b] Muramicitol, *N*-acetyl-muramicitol.
[c] Shown only for peaks subjected to amino acid analysis. The amino acid content and *N*-acetyl-muramicitol content are molar concentrations per mole of material in the peak. Glu, glutamic acid.

A comparison of the proposed cross bridges in the resistant strains, 18-73 and 18-88, and the susceptible parent, strain 18, was carried out on the basis of the observed masses in some peaks. Peaks 11, 13, 14, and 15 contain several comigrating muropeptides. Peak 11 for strain 18 contains structures with masses of 2,477 (11a), 2,457 (11b), and 2,507 (11c) Da (Fig. 3A), whereas peak 11 for strains 18-73 and 18-88 (Fig. 3B and C) contains, besides structures 11a and 11c, structures 11d and 11e, with monoisotopic masses of 2,249 and 2,537 Da, respectively (Fig. 4). Analysis of the deconvoluted mass spectrum of the material in peak 11 showed that for strain 18, muropeptides 11a, 11b, and 11c contribute 29, 46, and 25%, respectively, to the total, whereas for strain 18-73, muropeptides 11a, 11c, 11d, and 11e (no muropeptide 11b could be detected) contributed 19, 36, 25, and 20%, respectively, to the total peak material. Strains 18-73 and 18-88 have the muropeptide shown in peak 13d (Fig. 3B) in addition to those for 13a to 13c (Fig. 3A). The relative contributions of muropeptides 13a, 13b, and 13c to peak 13 in strain 18 are 39, 36, and 25%, respectively, while for strain 18-73, the relative contributions of muropeptides 13a, 13b, 13c, and 13d are 0 (undetectable), 22, 52, and 25%, respectively. Therefore, a muropeptide containing a cross-link with $Ser_2$ appeared in peaks 11 and 13 in strains 18-73 and 18-88 in the place of GlySer in strain 18 (Fig. 3). Quantitatively, this involved approximately 20% of the material in peak 11 and 25% of the material in peak 13. The position of the second serine was deduced on the basis of an analysis of precursors in *S. haemolyticus* strains in which a precursor bearing a GlySer partial cross bridge was detected (data not shown). Peaks 14 and 15, like peaks 11 and 13, each contained multiple comigrating muropeptides with different cross bridges. In this case the proposed structures are summarized in Table 3. Also shown in Table 3 is the contribution of each of the various mass signals to the total material in the peak. It appears that the predominant cross bridge in the muropeptide of peak 14 from strain 18 contains $Gly_2SerGly_2$, whereas strains 18-73 and 18-88 have a greater number of cross bridges containing $GlySer_2Gly_2$. The same phenomenon can be seen for the muropeptides in peak 15: strain 18 has cross bridges containing $Gly_2SerGly_2$, $AlaGly_4$, or $AlaGlySerGly_2$, whereas strains 18-73 and 18-88 have more cross bridges containing $GlySer_2Gly_2$ and $AlaGlySer_2Gly$ (although, in this case, the position of the second serine has not been established). In peak 14 of peptidoglycan from strain 18, no muropeptides contain $Ser_2$; in peak 14 from strain 18-73, 100% of the trimeric muropeptides contain at least one cross bridge with $Ser_2$. The situation is similar for peak 15, where only 32% of the muropeptides from strain 18 contain one cross bridge with $Ser_2$, while 100% of the trimeric muropeptides in peak 15 from strain 18-73 contain $Ser_2$ in at least one cross bridge. From the data presented in Table 2 and from our estimate of the contributions of each component from the deconvoluted mass spectra of peaks 11, 13, 14, and 15, we estimate that cross bridges containing $Ser_2$ have replaced GlySer in 10.5 to 13.6% of peptidoglycan in strain 18-73 as opposed to strain 18. Strain 18-88 is very similar to strain 18-73 except that there appear to be more lower-molecular-weight structures, as indicated by peaks E, F, G, and H (Fig. 3C). The position of these putative low-molecular-weight muropeptides, with the exception of muropeptide F, seems inappropriately late in the chromatogram, but the proposed structures account for the observed masses. These putative structures would, again, probably result from limited peptidoglycan breakdown. Muropeptides 3 to 5 are present in peptidoglycan of all strains.

**Competition of soluble glycopeptide target for vancomycin.** Soluble pentapeptide competes more effectively for vancomycin in the presence of strain 18-88 than in the presence of strain 18 (Fig. 5). This difference cannot be accounted for by the difference in vancomycin susceptibility of the two strains as demonstrated by our standard bioassay curves (data not shown). Identical experiments with teicoplanin (data not


A
G-M
Peak 1
Mo = 498
Mc = 498
M-Ala-GlNH$_2$-Lys-(Gly)$_5$-Ala
Peak 2
Mo = 976
Mc = 976
G-M-Ala-GlNH$_2$-Lys-(Gly)$_3$-Ala
Peak 3
Mo =1067
Mc = 1067
G-M-Ala-Glu-Lys-(Gly)$_5$-Gly-Ala
Peak 4a
Mo = 1239
Mc = 1239
G-M-Ala-Glu-Lys-(Gly)$_2$Ser(Gly)$_2$-Gly-Ala
Peak 4b
Mo = 1270
Mc = 1269
G-M-Ala-Glx-Lys-(Gly)$_2$Ser(Gly)$_2$-(Gly)$_2$-Ala
Peak 4c
Mo = 1326
Mc = 1326
G-M-Ala-Glu-Lys-(Gly)$_2$(Ser)$_2$Gly-(Gly)$_2$SerGly-Ala
Peak 4d
Mo = 1501
Mc = 1501
G-M-Ala-Glu-Lys-Gly-Ala-Ala
Peak 5
Mo = 1025
Mc = 1025
G-M-Ala-GlNH$_2$-Lys-(Gly)$_2$Ser(Gly)$_2$-Ala-Ala
Peak 6
Mo = 1282
Mc = 1282
G-M-Ala-GlNH$_2$-Lys-Ala-GlySer(Gly)$_2$-Ala-Ala
Peak 7
Mo = 1296
Mc = 1296
G-M-Ala-GlNH$_2$-Lys-Ala-GlySer(Gly)$_2$-Ala-Ala
G-M-Ala-GlNH$_2$-Lys-Ala-(Gly)$_2$
Peak 8
Mo = 2360
Mc = 2360
G-M-Ala-GlNH$_2$-Lys-Ala-GlySer(Gly)$_2$-Ala-Ala-Ala
G-M-Ala-GlNH$_2$-Lys-Ala-Gly-Ser
Peak 9
Mo = 2387
Mc = 2390
G-M-Ala-Glu-Lys-Ala-(Gly)$_4$-Lys
G-M-Ala-Glu
Peak 10
Mo = 1934
Mc = 1934
G-M-Ala-GlNH$_2$-Lys-(Gly)$_2$Ser(Gly)$_2$-Ala-Ala-Ala
G-M-Ala-Glu-Lys-(Gly)$_2$Ser(Gly)$_2$
Peak 11a
Mo = 2477
Mc = 2477
G-M-Ala-GlNH$_2$-Lys-(Gly)$_2$Ser(Gly)$_2$-Ala-Ala-Ala
G-M-Ala-GlNH$_2$-Lys-Ala(Gly)$_4$
Peak 11b
Mo = 2457
Mc = 2459
G-M-Ala-Glu-Lys-Ala-GlySer(Gly)$_2$-Ala-Ala-Ala
G-M-Ala-Glu-Lys-Ala-GlySer(Gly)$_2$
Peak 11c
Mo = 2507
Mc = 2505
G-M-Ala-Glu-Lys-AlaGly(Ser)$_2$Gly-Lys
G-M-Ala-Glu
Peak 12
Mo = 1994
Mc = 1994
G-M-Ala-GlNH$_2$-Lys-Ala(Gly)$_4$-Ala-Ala-Ala
G-M-Ala-GlNH$_2$-Lys-(Gly)$_2$Ser(Gly)$_2$
Peak 13a
Mo = 2461
Mc = 2460
G-M-Ala-GlNH$_2$-Lys-AlaGlySer(Gly)$_2$-Ala-Ala-Ala
G-M-Ala-GlNH$_2$-Lys-(Gly)$_2$Ser(Gly)$_2$
Peak 13b
Mo = 2491
Mc = 2490
G-M-Ala-GlNH$_2$-Lys-Ala-GlySer(Gly)$_2$-Ala-Ala-Ala
G-M-Ala-GlNH$_2$-Lys-Gly(Ser)$_2$(Gly)$_2$
Peak 13c
Mo = 2520
Mc = 2520
G-M-Ala-Glx-Lys-1-Ala-Ala
G-M-Ala-Glx-Lys-2-Ala
G-M-Ala-Glx-Lys-3
Peaks 14, 15

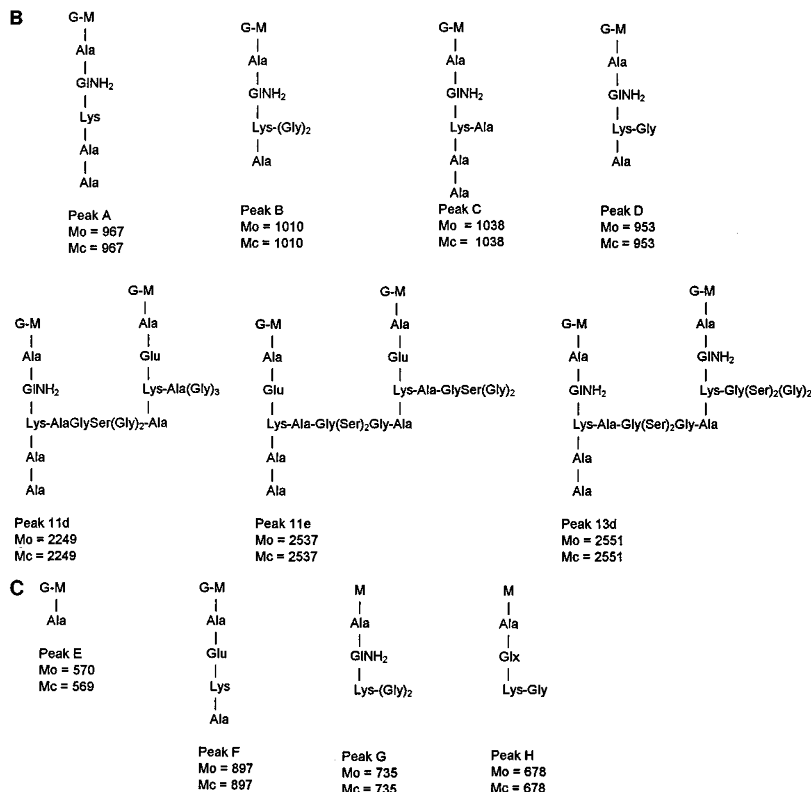



FIG. 3. (A) Structures proposed for muropeptide fragments of *S. haemolyticus* 18. (B) Structures proposed for muropeptide fragments unique to strain 18-73 compared with strain 18. Muropeptides B and C were found within the same peak. (C) Structures proposed for muropeptide fragments unique to strain 18-88 compared with strains 18 and 18-73. Muropeptides F and G were found within the same peak. In other instances in which more than one molecule has been identified within one peak, the structure has been given a lettered subdesignation (e.g., structures 4a and 4b). Subdesignations unique to one strain are shown as such. Cross bridges for peaks 14 and 15, designated as 1, 2, and 3, are described in Table 3. Mo, observed monoisotopic mass; Mc, calculated monoisotopic mass (both in daltons); G-M, GlcNAc-MurNAc; $GlNH_2$, glutamine.

shown) revealed no difference between the two strains in competition of soluble pentapeptide target for the antibiotic.

## DISCUSSION

Milewski et al. have now identified the 35- to 39-kDa membrane protein of glycopeptide-resistant *S. aureus* as a member of the D-alanine dehydrogenase family of enzymes (16) similar to the VanH protein of glycopeptide-resistant enterococci, which functions to convert pyruvate into D-lactate. In glycopeptide-resistant enterococci, the D-lactate is then incorporated into the UDP-muramyl precursor as D-alanyl-D-lactate in the place of D-alanyl-D-alanine, forming a depsipeptide resistant to glycopeptide binding (5). The data of Milewski et al. (16) suggested that an altered UDP precursor might also exist in resistant strains of staphylococci. Our data, unlike those of others (27), are consistent with their observations. Other workers have probably not detected this small amount of D-lactate-bearing precursor because the HPLC analysis alone is not sufficiently sensitive. Only with the aid of LC-MS were we able to detect this structure. However, it is not clear that 1.7% of total precursor as depsipeptide can explain the resistance of this strain. In one study of enterococcal strains (10), 10% of total precursor as depsipeptide increased the MIC of vancomycin from 2 to 32 μg/ml. In the case of *S. haemolyticus*, the MIC of vancomycin increases from 4 to 16 μg/ml with only 1.7% of precursor as depsipeptide.

Staphylococcal peptidoglycan is composed, as previously suggested by Schleifer and Kandler (20) and de Jonge et al. (7), of a lysine muropeptide acceptor containing at its ε-amino position a substitution of a glycine-containing cross bridge to the D-alanine 4 of the donor. We were unable to identify disaccharide-tetrapeptide-D-lactate as monomer or acceptor. This is not surprising, given the small amount of D-lactate-bearing precursor present and the somewhat limited sensitivity of the peptidoglycan analysis.

*S. haemolyticus* 18 and 18-73 contain, within their peptidoglycan, structures which appear to represent simply the dissaccharide (peaks with masses of 498 Da) which could result from an amidase-like activity. The peptidoglycan of *S. haemolyticus* 18 and 18-73 also contains structures which could be branched muramyl tetrapeptides (peak 2), which would result from a glucosaminidase activity. A glucosaminidase activity has been described for *S. aureus* (26).

In the case of *S. haemolyticus*, even the most susceptible strain of our series, strain 18, synthesizes several different cross bridges, the most common being $Gly_2SerGly_2$ or, less frequently, $AlaGlySerGly_2$. *S. haemolyticus*, like *fem* mutants of *S. aureus* (14), is less susceptible than *S. aureus* to digestion by lysostaphin (19, 22); this is probably explained by the presence of alanine and serine in place of glycine within the cross bridges of the peptidoglycan of these strains. Strains 18-73 and 18-88 have more cross bridges containing $GlySer_2Gly_2$ and $AlaGlySer_2Gly$. Although we have been able to analyze only up to half of the muropeptides of the peptidoglycan of these strains, our data might indicate that as much as 13.6% or more

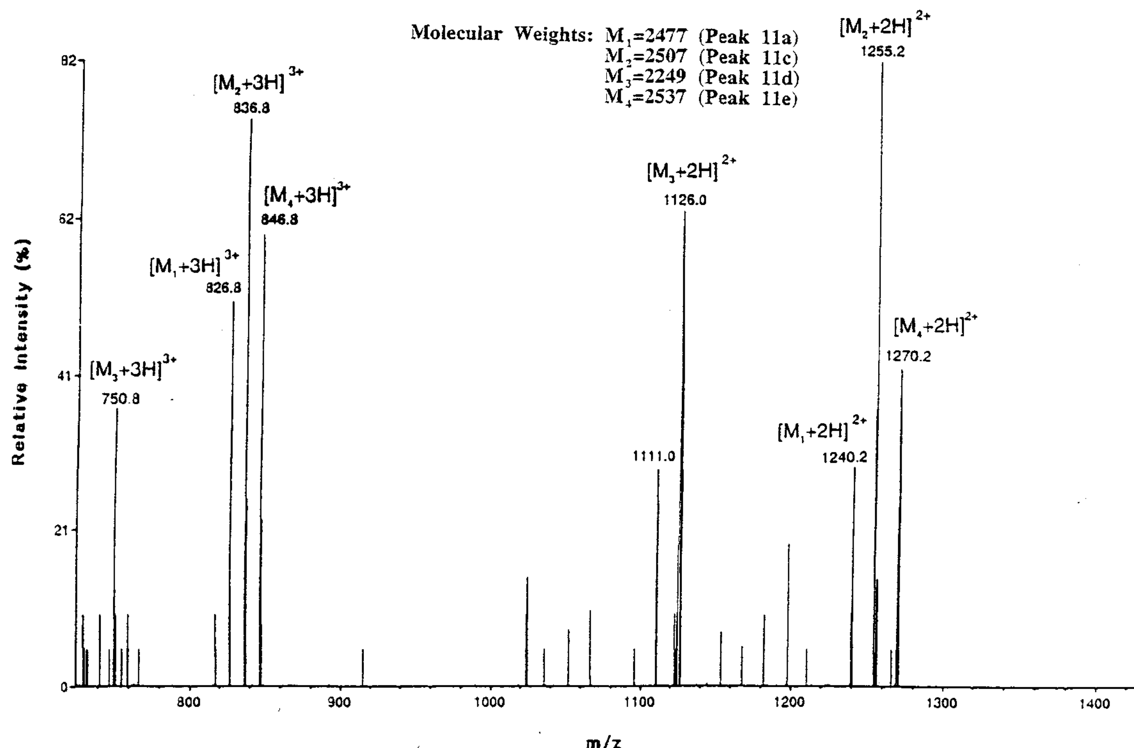



FIG. 4. Mass spectrum of peak 11 of the peptidoglycan of *S. haemolyticus* 18-73. The molecular weights shown are monoisotopic.

of muropeptides from resistant strains may contain these altered cross bridges (Tables 2 and 3). Since strain 18-73 does not demonstrate altered expression of penicillin-binding proteins (22), the presence of additional serines in the cross bridge cannot be related simply to altered penicillin-binding protein expression.

At the same time, we examined the contribution of cooperative glycopeptide binding to antibiotic activity in these strains. These experiments were based on those recently described by Beauregard et al. (3). We observed that the soluble pentapeptide target was more effective at competing for the binding of vancomycin in the presence of the resistant strain than in the presence of the susceptible strain. Experiments with teicoplanin demonstrated no difference in target competition for the two strains. Vancomycin is thought to act partly via dimerization and cooperative binding, while teicoplanin is thought not to dimerize but rather to rely on a membrane anchor for stable binding. Our data suggest that resistance is directed, in part, towards the binding of vancomycin dimers to target (and, therefore, cooperative binding) in situ. Whether the altered peptidoglycan cross bridge can explain this by interference with vancomycin dimerization and binding in situ is as yet unknown.

TABLE 3. Cross bridges proposed for muropeptides in peaks 14 and 15 of *S. haemolyticus*

| Peaks and strains | Mass (Da) | Cross bridge[a] | | | Molecular abundance (%)[b] |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | |
| Peak 14 | | | | | |
| Strain 18 | 3,641 | $Gly_5$ | $Gly_2SerGly_2$ | $Gly_2SerGly_2$ | 58 |
| | 3,670 | $Gly_2SerGly_2$ | $Gly_2SerGly_2$ | $Gly_2SerGly_2$ | 42 |
| Strains 18-73 and 18-88 | 3,705 | $Gly_2SerGly_2$ | $Gly_2SerGly_2$ | $GlySer_2Gly_2$ | 36 |
| | 3,735 | $Gly_2SerGly_2$ | $GlySer_2Gly_2$ | $GlySer_2Gly_2$ | 36 |
| | 3,765 | $GlySer_2Gly_2$ | $GlySer_2Gly_2$ | $GlySer_2Gly_2$ | 28 |
| Peak 15 | | | | | |
| Strain 18 | 3,627 | $Gly_5$ | $Gly_2SerGly_2$ | $AlaGly_4$ | 14 |
| | 3,657 | $Gly_2SerGly_2$ | $Gly_2SerGly_2$ | $AlaGly_4$ | 28 |
| | 3,687 | $Gly_2SerGly_2$ | $Gly_2SerGly_2$ | $AlaGlySerGly_2$ | 25 |
| | 3,717 | $Gly_2SerGly_2$ | $GlySer_2Gly_2$ | $AlaGlySerGly_2$ | 32 |
| Strains 18-73 and 18-88 | 3,717 | $Gly_2SerGly_2$ | $GlySer_2Gly_2$ | $AlaGlySerGly_2$ | 40 |
| | 3,747 | $GlySer_2Gly_2$ | $GlySer_2Gly_2$ | $AlaGlySerGly_2$ | 27 |
| | 3,777 | $GlySer_2Gly_2$ | $GlySer_2Gly_2$ | $AlaGlySer_2Gly$ | 33 |

[a] Structures are shown in the following order: acceptor to donor, carboxyl to amino terminus, from left to right, as illustrated in Fig. 3A. The order of cross bridges 1, 2, and 3 is arbitrary.

[b] Determined by analysis of peak heights from deconvoluted mass spectra of peptidoglycan from strains 18 and 18-73.

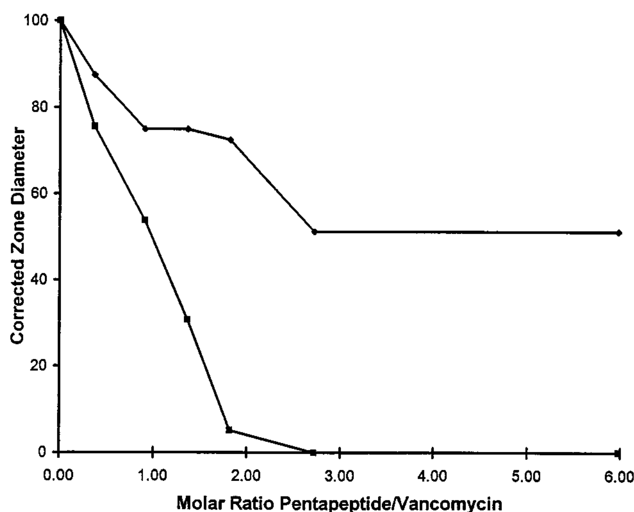



FIG. 5. Competition of pentapeptide for vancomycin activity against *S. haemolyticus*. ◆, strain 18; ■, strain 18-88. Data are plotted as the zone diameters around a 7-mm-diameter disk (corrected to 100% for the sample with no added pentapeptide).

Since teicoplanin does not bind its target in a cooperative manner, these experiments cannot address resistance to this glycopeptide in these strains.

In conclusion, it is possible that glycopeptide resistance in *S. haemolyticus* is multifactorial in nature and that both the presence of UDP-muramyl-tetrapeptide-D-lactate cytoplasmic precursor and altered peptidoglycan cross bridges contribute to this resistance.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## REFERENCES


1. **Arthur, M., F. Depardieu, C. Molinas, et al.** 1995. The *vanZ* gene of Tn*1546* from *Enterococcus faecium* BM4147 confers resistance to teicoplanin. Gene **154:**87–92.
2. **Arthur, M., C. Molinas, F. Depardieu, and P. Courvalin.** 1993. Characterization of Tn*1546*, a Tn*3*-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. **175:**117–127.
3. **Beauregard, D. A., D. H. Williams, M. N. Gwynn, and D. J. C. Knowles.** 1995. Dimerization and membrane anchors in extracellular targeting of vancomycin group antibiotics. Antimicrob. Agents Chemother. **39:**781–785.
4. **Billot-Klein, D., L. Gutmann, S. Sablé, E. Guittet, and J. van Heijenoort.** 1994. Modification of peptidoglycan precursors is a common feature of the low-level vancomycin-resistant VANB-type enterococcus D366 and of the naturally glycopeptide-resistant species *Lactobacillus casei*, *Pediococcus pentosaceus*, *Leuconostoc mesenteroides*, and *Enterococcus gallinarum*. J. Bacteriol. **176:**2398–2405.
5. **Bugg, T. D. H., G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh.** 1991. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistant proteins VanH and VanA. Biochemistry **30:**10408–10415.
6. **Daum, R. S., S. Gupta, R. Sabbagh, and W. M. Milewski.** 1992. Characterization of *Staphylococcus aureus* isolates with decreased susceptibility to vancomycin and teicoplanin; isolation and purification of a constitutively produced protein associated with decreased susceptibility. J. Infect. Dis. **166:**1066–1072.
7. **de Jonge, B. L. M., Y. S. Chang, D. Gage, and A. Tomasz.** 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin-binding protein 2A. J. Biol. Chem. **267:**11248–11254.
8. **Glauner, B.** 1988. Separation and quantification of muropeptides with high-performance liquid chromatography. Anal. Biochem. **172:**451–464.
9. **Greenwood, D.** 1988. Microbiological properties of teicoplanin. J. Antimicrob. Chemother. **21**(Suppl. A)**:**1–13.
10. **Handwerger, S.** 1994. Alterations in peptidoglycan precursors and vancomycin susceptibility in Tn*917* insertion mutants of *Enterococcus faecalis* 221. Antimicrob. Agents Chemother. **38:**473–475.
11. **Herwaldt, L., L. Boyken, and M. Pfaller.** 1991. In vitro selection of resistance to vancomycin in bloodstream isolates of *Staphylococcus hemolyticus* and *Staphylococcus epidermidis*. Eur. J. Clin. Microbiol. Infect. Dis. **10:**1007–1012.
12. **Kaatz, G. W., S. M. Seo, N. J. Dorman, and S. A. Lerner.** 1990. Emergence of teicoplanin resistance during therapy of *Staphylococcus aureus* endocarditis. J. Infect. Dis. **162:**103–108.
13. **Mackay, J. P., U. Gerhard, D. A. Beauregard, R. A. Maplestone, M. S. Westwell, M. S. Searle, and D. H. Williams.** 1994. Glycopeptide antibiotic activity and the possible role of dimerization: a model for biological signaling. J. Am. Chem. Soc. **116:**4581–4590.
14. **Maidhof, H., B. Reinicke, P. Blümel, B. Berger-Bächi, and H. Labischinski.** 1991. *femA*, which encodes a factor essential for expression of methicillin resistance, affects glycine content of peptidoglycan in methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. J. Bacteriol. **173:**3507–3513.
15. **Mainardi, J. L., D. M. Shlaes, R. V. Goering, J. H. Shlaes, J. F. Acar, and F. W. Goldstein.** 1995. Decreased teicoplanin susceptibility of methicillin-resistant strains of *Staphylococcus aureus*. J. Infect. Dis. **171:**1646–1650.
16. **Milewski, W. M., S. Boyle-Vavra, B. Moreira, C. C. Ebert, and R. S. Daum.** 1996. Overproduction of a 37-kilodalton cytoplasmic protein homologous to $NAD^+$-linked D-lactate dehydrogenase associated with vancomycin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **40:**166–172.
17. **Noble, W. C., Z. Virani, and R. G. A. Gee.** 1992. Co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*. FEMS Microbiol. Lett. **93:**195–198.
18. **O'Hare, M. D., and P. E. Reynolds.** 1992. Novel membrane proteins present in teicoplanin-resistant vancomycin sensitive coagulase negative *Staphylococcus* spp. J. Antimicrob. Chemother. **30:**753–768.
19. **Recsei, P. A., A. D. Gruss, and R. P. Novick.** 1987. Cloning, sequence and expression of the lysostaphin gene from *Staphylococcus simulans*. Proc. Natl. Acad. Sci. USA **84:**1127–1131.
20. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36:**407–477.
21. **Schwalbe, R. S., J. T. Stapleton, and P. H. Gilligan.** 1987. Emergence of vancomycin resistance in coagulase negative staphylococci. N. Engl. J. Med. **36:**927–931.
22. **Shlaes, D. M., and L. Herwaldt.** Unpublished observations.
23. **Shlaes, D. M., and J. H. Shlaes.** 1995. Teicoplanin selects for *Staphylococcus aureus* that is resistant to vancomycin. Clin. Infect. Dis. **20:**1071–1073.
24. **Shlaes, D. M., J. H. Shlaes, S. Vincent, L. Etter, P. D. Fey, and R. V. Goering.** 1993. Teicoplanin-resistant *Staphylococcus aureus* expresses a novel membrane protein and increases expression of penicillin-binding protein 2 complex. Antimicrob. Agents Chemother. **37:**2432–2437.
25. **Somner, E. A., and P. E. Reynolds.** 1990. Inhibition of peptidoglycan biosynthesis by ramoplanin. Antimicrob. Agents Chemother. **34:**413–419.
26. **Sugai, M., H. Komatsuzawa, T. Akiyama, Y.-M. Hong, T. Oshida, Y. Miyake, T. Yamaguchi, and H. Suginaka.** 1995. Identification of endo-β-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase as cluster-dispersing enzymes in *Staphylococcus aureus*. J. Bacteriol. **177:**1491–1496.
27. **Verma, P. R., N. E. Allen, J. N. Hobbs, Jr., J. A. Johnson, J. G. Morris, Jr., and R. S. Schwalbe.** 1994. Characterization of vancomycin resistance in *Staphylococcus haemolyticus*, abstr. C47, p. 76. *In* Program and abstracts of the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy. American Society for Microbiology, Washington, D.C.