High Level Oxacillin and Vancomycin Resistance and Altered Cell Wall Composition in Staphylococcus aureus Carrying the Staphylococcal mecA and the Enterococcal vanA Gene Complex*

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Recently, for the first time in the history of this bacterial species, methicillin-resistant Staphylococcus aureus (MRSA) carrying the enterococcal vanA gene complex and expressing high level resistance to vancomycin was identified in clinical specimens (CDC (2002) MMWR 51, 565–567). The purpose of our studies was to understand how vanA is expressed in the heterologous background of S. aureus and how it interacts with the mecA-based resistance mechanism, which is also present in these strains and is targeted on cell wall biosynthesis. The vanA-containing staphylococcal plasmid was transferred from the clinical vancomycin-resistant S. aureus (VRSA) strain HIP11714 (CDC (2002) MMWR 51, 565–567) to the methicillin-resistant S. aureus (MRSA) strain COL for which extensive genetic and biochemical information is available on staphylococcal cell wall biochemistry and drug resistance mechanisms. The transconjugant named COLVA showed high and homogeneous resistance to both oxacillin and vancomycin. COLVA grown in vancomycin-containing medium produced an abnormal peptidoglycan: all pentapeptides were replaced by tetrapeptides, and the peptidoglycan contained at least 22 novel muropeptide species that frequently showed a deficit or complete absence of pentaglycine branches. The UDP-MurNAc-pentapeptide, the major component of the cell wall precursor pool in vancomycin-sensitive cells was replaced by UDP-MurNAc-tetrapeptide and UDP-MurNAc-tetrapentapeptide. Transposon inactivation of the β-lactam resistance gene mecA caused complete loss of β-lactam resistance but had no effect on the expression of vancomycin resistance. The two major antibiotic resistance mechanisms encoded by mecA and vanA residing in the same S. aureus appear to use different sets of enzymes for the assembly of cell walls.

Until the late 1990s, clinical isolates of Saccharomyces cerevisiae have retained a uniform high sensitivity to vancomycin with minimal inhibitory concentration (MIC) values in the vicinity of 1 μg/ml. Beginning with the late 1990s, isolates with reduced susceptibility to vancomycin began to be reported from several countries (1), but the MIC value of these vancomycin intermediate-resistant S. aureus isolates was limited to the range of 8–16 μg/ml of the antibiotic. The first highly vancomycin-resistant S. aureus (VRSA) isolates (MIC over 32 μg/ml) were only detected during the last year in two hospitals in the United States (2, 3). The appearance of such VRSA strains in clinical specimens is of obvious and grave concern, because the spread of VRSA isolates may seriously jeopardize the chemotherapy of multidrug-resistant S. aureus disease and raise the specter of untreatable staphylococcal infections (4, 5).

The VRSA strain HIP11714 recovered from a dialysis patient in Detroit, Michigan has acquired the vanA gene complex most likely from a vancomycin-resistant Enterococcus faecalis strain confecting the diabetic wound and catheter insertion site of the patient (1). HIP11714 was also methicillin-resistant: it carried the heterologous mecA gene, the key component of wide spectrum β-lactam resistance. Clinical aspects of this case (1), the genetic background of HIP11714 (4), and the structure of the S. aureus plasmid pLW1043 into which the transposon Tn1546 integrated2 are the subject of separate publications. The purpose of the study described here was different: we wanted to examine how two very different antibiotic resistance mechanisms, based either on mecA or on vanA, are expressed in the same S. aureus cell and what impact the enterococcal vanA gene complex may have on the structure of the staphylococcal cell wall. The mechanism of vanA-mediated vancomycin resistance, at least when it is expressed in enterococci, involves the production of a cell wall precursor with abnormal chemical structure (for recent review see Ref. 6). The mechanism of mecA-based β-lactam resistance involves the production of a new cell wall biosynthetic enzyme, the penicillin-binding protein 2A (PBP2A) (7–9). To understand interactions between these two resistance mechanisms each targeting the bacterial cell wall, we transferred the vanA-carrying plasmid from the clinical strain HIP1174 to the MRSA strain COL, in which extensive biochemical and genetic information on cell wall

diphospho-N-acetylmuramy1-L-Ala-t-Glu-t-Lys-t-Ala; UDP-MurNac-depsipeptide, uridinephospho-N-acetylamuramyl1-L-Ala-t-Glu-t-Lys-t-Ala-t-Lac; MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylgalactosamine; Lipid II, undecaprenylpyrophosphoryl-N-acetylmuramyl-(peptide)-N-acetylgalactosamine; MS/MS, fragmentation analysis by mass spectrometry; LC-MS, liquid chromatography (HPLC) coupled to mass spectrometry; HPLC, high performance liquid chromatography; PBP, penicillin-binding protein; VRSA, vancomycin-resistant S. aureus; MRSA, methicillin-resistant S. aureus; BHI, brain heart infusion.

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¶ The abbreviations used are: MIC, minimal inhibition concentration; UDP-MurNAc-pentapeptide, uridinephospho-N-acetylamuramyl1-L-Ala-t-Glu-t-Lys-t-Ala-t-Ala; UDP-MurNAc-tetrapeptide, uridinephospho-N-acetylmuramyl-L-Ala-t-Glu-t-Lys-t-Ala-t-Ala; BHI, brain heart infusion.
Expression of vanA in S. aureus

EXPERIMENTAL PROCEDURES

Construction of Strain COLVA by Filter Mating—In the filter mating experiments the recipient used was the MRSA strain COL, and the donor was the clinical VRSA isolate HIP11714, which carried transposon Tn551-inactivated mecA (C and D) were assayed for the level of resistance to oxacillin and vancomycin. Overnight cultures of bacterial strains grown in TSB (A and C) or in TSB containing 100 μg/ml vancomycin (B and D) were diluted and spread on TSA after which the antibiotic containing paper strips (oxacillin strips in A and C and vancomycin strips in B and D) were deposited on the agar surface.

structure (10–12) and biosynthesis (13–15) is available. Strain COL does not carry chromosomal or plasmid-born regulatory elements that often control the transcription of mecA in clinical MRSA isolates (16, 17). Strain COL is one of the fully sequenced MRSA strains (18) in which a large body of genetic information is also available concerning genetic determinants, auxiliary genes (19–22), that are essential for the optimal expression of β-lactam resistance. These genetic determinants may provide useful tools for the dissection of the vanA- and mecA-mediated resistance mechanisms.

Testing Antibiotic Susceptibility—Antibiotic resistance level and synergistic activity of antibiotics were determined by the E-test following the recommendations by the manufacturer (Ab Biodisk, Solna, Sweden) and by the method of population analysis (23).

Preparation and Analysis of Peptidoglycan—Cell wall peptidoglycan was prepared, purified, and solubilized by enzymatic hydrolysis using previously described methods (10, 11, 13). The muropeptides liberated by the enzymatic hydrolysis were reduced by borohydride and separated by reversed-phase high performance liquid chromatography (HPLC) with a Shimadzu LC-10A HPLC system as described before (13). The peaks of interest were isolated and desalted by HPLC (13), and MS analysis was performed.

Preparation of UDP-linked Peptidoglycan Precursors and Analysis by HPLC—Cytosolic pools of the UDP-linked precursors were extracted by a method previously described (24) with some modification (25). In strains COL and COLVA grown in antibiotic-free medium, bacitracin (100 μg/ml or 2× MIC) was added to the cultures 30 min before harvesting the cells to amplify the amounts of cell wall precursors. Recent experiments in our laboratory have shown that bacitracin, an inhibitor of the regeneration of bactoprenylphosphate in the membrane transfer reaction of cell walls synthesis (26, 27) can be successfully used instead of vancomycin (26) for the amplification of the cell wall precursor pool. Analysis was performed with a Shimadzu LC-10A HPLC system. Samples were applied to a 250×4.6-mm reversed-phase HPLC column (ODS-Hypersil, 3 μm, Keystone Scientific, Bellefonte, PA). The column was eluted at a flow rate of 0.5 ml/min with a linear gradient of 5% (v/v) of acetonitrile in water at a flow rate of 0.5 ml/min.

Expression of vanA in S. aureus

Strains and Growth Conditions—S. aureus strains were grown in tryptic soy broth (TSB) (Difco) at 37 °C with aeration. Growth was monitored by measuring the optical density at 600 nm with an LKB spectrophotometer. In some experiments, peptidoglycan and cell wall precursor analyses were performed on bacteria grown in brain heart infusion (BHI) broth (Difco, Detroit, MI) were diluted and spread on TSA after which the bacteria were eluted into BHI broth and plated on agar containing 50 μg/ml tetracycline (selective marker for the recipient). One of the five transconjugants was purified by two consecutive single colony isolations on the selective agar and was transferred to the Laboratory at the Rockefeller University where the purity and stability of the drug resistance trait was retested and the transconjugant was named strain COLVA.

Fig. 1. Expression of high level oxacillin and vancomycin resistance in S. aureus. S. aureus strain COLVA carrying both mecA and vanA (A and B) and a mutant derivative COLVAΔmecA with a Tn551-inactivated mecA (C and D) were assayed for the level of resistance to oxacillin and vancomycin. Overnight cultures of bacterial strains grown in TSB (A and C) or in TSB containing 100 μg/ml vancomycin (B and D) were diluted and spread on TSA after which the antibiotic containing paper strips (oxacillin strips in A and C and vancomycin strips in B and D) were deposited on the agar surface.

Fig. 2. Differences in the muropeptide composition of the vancomycin resistant S. aureus strain COLVA grown in antibiotic-free and in vancomycin-containing medium. Strain COLVA was grown in antibiotic-free TSB (B), TSB containing 250 μg/ml vancomycin (C), and in TSB containing 10 μg/ml oxacillin (D). As control strain COL was grown in TSB free of antibiotic (A). Cell wall peptidoglycan was prepared and enzymatic hydrolysates separated by HPLC. The numbers refer to main muropeptide components of strain COL (13), and the numbers followed by the letter v refer to the novel muropeptide components of the vancomycin-resistant strain COLVA. The arrows in B indicate muropeptide species that become dominant in the vancomycin grown bacteria (C).
methanol in 100 mM ammonium formate (pH 3.5) to 30% (v/v) methanol in 100 mM ammonium formate (pH 3.5) in 30 min. The pH of buffers was adjusted by formic acid. Column temperature was 40 °C.

The flow rate was 0.5 ml/min for the HPLC separation, and it was reduced to 50 ml/min before the ion source. To improve the separation of early precursors (UDP-MurNAc and UDP-MurNAc-Ala) the method was modified: the pH of the 100 mM ammonium formate was adjusted to pH 2.5 by HCl.

Mass Spectrometry Analysis—Samples of muropeptides were isolated by HPLC, lyophilized, and dissolved in H₂O:CH₃CN (50:50, v/v). A sample was injected at a flow rate of 50 µl/min into a Micromass quadrupole time-of-flight electrospray mass spectrometer operating in the positive ion mode. A MS/MS analysis (31, 32) of UDP-MurNAc-1-Ala-γ-3-Glu-Lys-D-Ala-D-Lac and MurNAc-L-Ala-D-Glu-L-Lys-D-Ala-D-Lac moieties of the molecules. The latter fragment ion was subjected to an additional stage of MS/MS analysis. Table I shows the experimental and theoretical molecular masses obtained for the 22 new muropeptide species detected. The proposed chemical structures derived from the data are shown in Fig. 3. The most striking and novel features of these muropeptides are (i) the complete replacement of pentapeptides by tetrapeptides and (ii) the frequent deficit and/or complete lack of pentaglycine branches in muropeptides particularly in muropeptide monomers and in the biosynthetically “first” donor components of oligomeric muropeptides. Also apparent were structural modifications in some muropeptides that we interpret as evidence for lack of amidation of the stempeptide glutamic acid residues (muropeptide 4v) and the parent were structural modifications in some muropeptides.

### RESULTS

**Expression of High Level Resistance to Oxacillin and Vancomycin in Strain COLVA**—Strain COLVA grown overnight either in TSB or in TSB supplemented with 100 µg/ml vancomycin were tested for susceptibility to oxacillin and vancomycin by the E-test. The cultures grown in TSB showed high level resistance to oxacillin (MIC, 800 µg/ml) (Fig. 1A) and the culture grown in vancomycin showed high level vancomycin resistance (MIC: 512 µg/ml) (Fig. 1B).

**Composition of the Cell Wall Peptidoglycan of Strain COLVA Grown in Antibiotic-free and Antibiotic-containing Media**—The HPLC elution profile of muropeptides recovered from the peptidoglycan of strain COLVA was compared with that of strain COLVA with both strains grown in antibiotic-free TSB. The two elution profiles were virtually identical, except that in strain COLVA next to many of the familiar muropeptide peaks of strain COL (13) (Fig. 2, top panel) there was often a small secondary peak eluting from the HPLC column with slightly shorter retention times (see arrows in Fig. 2, second panel). In COLVA grown in the presence of vancomycin these novel peaks became the dominant muropeptide species of the peptidoglycan (Fig. 2, third panel). Growth of COLVA in the presence of oxacillin (10 µg/ml) caused a virtual disappearance of all but one (peak 3v) of the novel peaks and the peptidoglycan composition changed to a profile characteristic of strain COL grown in the presence of β-lactam antibiotics (13) (Fig. 2, bottom panel).

**Structure of the Muropeptide Components in Strain COLVA Grown in Vancomycin-containing Medium**—The novel muropeptide peaks that became dominant in the vancomycin-grown cells were purified and subjected to mass spectrometric analysis. Table I shows the experimental and theoretical molecular masses obtained for the 22 new muropeptide species detected. The proposed chemical structures derived from the data are shown in Fig. 3. The most striking and novel features of these muropeptides are (i) the complete replacement of pentapeptides by tetrapeptides and (ii) the frequent deficit and/or complete lack of pentaglycine branches in muropeptides particularly in muropeptide monomers and in the biosynthetically “first” donor components of oligomeric muropeptides. Also apparent were structural modifications in some muropeptides that we interpret as evidence for lack of amidation of the stempeptide glutamic acid residues (muropeptide 4v) and the presence of deacetylated (16v) and O-acetylated (6v) hexosamines. The percent representation of various muropeptide peaks in bacteria grown under different conditions is illustrated in Table II.

**Composition of the Cell Wall Precursor Pool**—Fig. 4 shows the composition of the cytoplasmic cell wall precursor pool in strains COL and COLVA grown either in antibiotic-free medium or in media supplemented with antibiotics. These are the same growth conditions that were tested for their effect on the composition of the peptidoglycan in Fig. 2. In strains COL and COLVA grown in antibiotic-free TSB, the HPLC separation combined with mass spectrometric analysis identified six components in the precursor pool that were common and were
present in comparable amounts in both strains (see peaks 1–4, 6, and 7 in Fig. 4, first and second panels). An additional minor component (peak 8) was only detected in strain COLVA. Peaks 1–4 and 6 were identified as the well known cell wall muropeptide precursors: UDP-MurNAc (peak 1), UDP-MurNAc-L-Ala (peak 2), UDP-MurNAc-L-Ala-D-Glu-L-Lys (peak 3), UDP-

**FIG. 3.** Chemical structures of novel muropeptide components produced by the vancomycin-resistant strain COLVA. The proposed chemical structures were derived from the HPLC retention times and mass spectrometric analysis (see Table I).

**TABLE II**

| Major peak | Relative amounts of muropeptides* |
|------------|----------------------------------|
|            | No antibiotics | COLVA | COLVA + vancomycin | COLVA + oxacillin | COLVA – mecA + vancomycin |
| 1          | 1.4              | 1.7   | 0.0               | 1.6              | 0.0                       |
| 5          | 3.7              | 2.9   | 0.0               | 15.8             | 0.0                       |
| 11         | 5.4              | 4.3   | 0.0               | 12.6             | 0.0                       |
| 15         | 5.3              | 4.2   | 0.0               | 7.2              | 0.0                       |
| 16         | 3.7              | 3.2   | 0.0               | 3.8              | 0.0                       |
| 17         | 3.7              | 3.0   | 0.0               | 2.6              | 0.0                       |
| 3v         | 0.0              | 0.5   | 12.4             | 4.7              | 12.4                      |
| 8v         | 0.0              | 1.7   | 2.4              | 0.0              | 8.9                       |
| 11v        | 0.0              | 0.5   | 2.5              | 0.0              | 3.1                       |
| 13v        | 0.0              | 1.7   | 3.9              | 0.0              | 7.7                       |
| 14v        | 0.0              | 0.0   | 2.2              | 0.0              | 2.7                       |
| 15v        | 0.0              | 1.2   | 3.6              | 0.0              | 5.7                       |
| 16v        | 0.0              | 0.0   | 1.5              | 0.0              | 1.3                       |
| 17v        | 0.0              | 0.8   | 2.7              | 0.0              | 2.8                       |
| 19v        | 0.0              | 0.0   | 1.5              | 0.0              | 1.8                       |
| Monomers   | 13.2             | 13.9  | 24.4             | 35.2             | 35.3                      |
| Oligomers  | 48.8             | 47.6  | 38.2             | 53.7             | 48.8                      |
| Hump       | 38.0             | 38.5  | 37.4             | 11.1             | 15.8                      |

* Relative amounts of muropeptide species are expressed as the percent of total UV-absorbing material recovered from the HPLC column.
MurNAc-L-Ala-d-Glu (peak 4), and the pentapeptide derivative of UDP-MurNAc, UDP-MurNAc-L-Ala-d-Glu-Lys-D-Ala-d-Ala (peak 6) (35), the latter representing close to 80% of the precursor material. An additional minor peak 7 was identified in both COL and COLVA as a derivative of the UDP-MurNAc-pentapeptide carrying a single glycine substitute on the ε-amino group of the lysine residue. The precursor pool of COLVA (but not COL) also contained an additional minor component (peak 8), which had HPLC retention time and molecular mass characteristic of the UDP-MurNAc-depsipeptide, UDP-MurNAc-L-Ala-d-Glu-Lys-d-Ala-d-Lac, described in vancomycin-resistant enterococci (36, 37).

Radically different results were obtained when the cell wall precursor pool was analyzed from COLVA grown in vancomycin-containing medium (Fig. 4, third panel). Although peaks 1–4 appeared to be present unchanged, peak 6, the major component in the bacteria grown without vancomycin, was diminished and was replaced by two new major components (peaks 5 and 8). In addition several minor components were also present. Mass spectrometric analysis of peak 5 identified it as the UDP-MurNAc-L-Ala-d-Glu-Lys-d-Ala tetrapeptide. Peak 8 had a retention time of 21 min on the HPLC elution profile and a molecular mass that differed by 1 mass unit from that of the UDP-MurNAc-pentapeptide.

The composition of the cell wall precursor pool in strain COLVA grown in TSB supplemented with 10 μg/ml oxacillin (Fig. 4, bottom panel) was indistinguishable from the precursor profile of COLVA grown in drug-free TSB, including even the presence of small amounts of the UDP-MurNAc-depsipeptide (peak 8). The percentage representation of various compounds in the cytoplasmic cell wall precursor pool in COL and COLVA grown under different conditions is shown in Table III.

**Confirming the Structure of UDP-MurNAc-depsipeptide by MS/MS Analysis—LC/MS Analysis (29, 30) running in the positive ion mode showed that the peak 8 at retention time 21.0 min (Fig. 4) has a molecular ion mass [M+H]+ of m/z 1151.6. This molecular ion matched the predicted value of 1150.35 Da for UDP-MurNAc-L-Ala-γ-d-Glu-Lys-d-Ala-d-Lac. To provide a rigorous proof for the proposed structure, MS/MS analysis of the molecular ion was performed (Fig. 5).

Fragmentation of the molecular ion at m/z 1151.6 gave two product ions at m/z 747.5 and 562.4 that corresponded to the loss of UDP and UDP-GlcNAc from the precursor ion, respectively (Fig. 5, A and C). The ion at m/z 562.4 corresponds to the structure Lac-γ-d-Glu-Lys-d-Ala-d-Lac. The first lactate at the N-terminal in this fragment came from the cleavage of muramic acid between N-acetylglucosamine and lactate.

To obtain the sequence of the peptide, further fragmentation (Fig. 5B) of the ion at m/z 562.4 was performed by generating the precursor ion at the nozzle skimmer (33, 34) with a high cone voltage of 50 V. The fragmentation from the C-terminal generated a series of product ions at m/z 472.4, 401.4, 273.2, and 144.1 corresponding to the loss of d-Lac, d-Ala-d-Lac, L-Lys-d-Ala-d-Lac, and d-Glu-i-Lys-d-Ala-d-Lac, respectively. Another series of ions were also formed due to the fragmentation from the N-terminal. Those were m/z 490.4, 419.3, and 290.3 corresponding to the structures i-Ala-γ-d-Glu-Lys-d-Ala-d-Lac, d-Glu-i-Lys-d-Ala-d-Lac, and i-Lys-d-Ala-d-Lac, respectively.

The peaks at m/z 329.3 and 258.2 can be assigned to the structures i-Glu-i-Lys-d-Ala (possibly i-Ala-γ-d-Glu-Lys) and d-Glu-i-Lys, respectively. The last two structures suggest simultaneous fragmentation of lactylpentapeptide from both the N and C termini. Finally, the loss of H₂O and CO from the ions described above can explain the appearance peaks at m/z 544.4, 383.3, 240.2, and 116.1. The peak at m/z 195.1 may be

![Fig. 4. Composition of the cytoplasmic cell wall precursor pool in strains COL and COLVA grown in different medium. Bacterial cultures were grown, harvested, and extracted as described under "Experimental Procedures" and the composition of cell wall precursors was analyzed. The numbers refer to the following UDP-MurNAc derivatives: 1, UDP-MurNAc; 2, UDP-MurNAc-Ala; 3, UDP-MurNAc-Ala-Glu-Lys; 4, UDP-MurNAc-Ala-Glu; 5, UDP-MurNAc-Ala-Glu-Lys-Ala; 6, UDP-MurNAc-Ala-Glu-Lys-Ala-Ala; 7, UDP-MurNAc-Ala-Glu-Lys(Lys)Ala-Ala; 8, UDP-MurNAc-Ala-Glu-Lys-Lys-Lac; and 9, UDP-MurNAc-Ala-Glu-Lys(Lys)Ala-Lac.](http://www.jbc.org/)

**Table III Composition of cell wall precursor pool in the vancomycin-susceptible strain COL and vancomycin-resistant strain COLVA**

| Peak number | Precursor | No antibiotics COL | COL | COLA | COLA + vancomycin | COLA + oxacillin | COLA-ΔmecA + vancomycin |
|-------------|-----------|--------------------|-----|------|-------------------|-----------------|------------------------|
| 1 | UDP-MurNAc | 16.7 | 13.8 | 7.6 | 14.6 | 13.5 |
| 2 | UDP-MurNAc-Ala | 21.1 | 17.1 | 7.9 | 19.1 | 18.8 |
| 3 and 4 | UDP-MurNAc-Ala-Glu-Lys + UDP-MurNAc-Ala-Glu-Lys-Ala | 1.7 | 2.6 | 0.3 | 2.9 | 3.1 |
| 5 | UDP-MurNAc-Ala-Glu-Lys-Ala | 0.0 | 0.0 | 27.0 | 0.0 | 28.9 |
| 6 | UDP-MurNAc-Ala-Glu-Lys-Lys-Ala | 0.0 | 0.0 | 61.5 | 0.0 | 58.6 |
| 7 | UDP-MurNAc-Ala-Glu-Lys(Lys)Ala-Ala | 1.3 | 1.3 | 0.0 | 1.4 | 0.0 |
| 8 | UDP-MurNAc-Ala-Glu-Lys-Lys-Lac | 0.0 | 0.0 | 34.6 | 1.3 | 39.0 |
| 9 | UDP-MurNAc-Ala-Glu-Lys(Lys)Ala-Lac | 0.0 | 0.0 | <1 | 0.0 | 0.0 |
| Total | | 98 | 98 | 79 | 98 | 95 |

* To accumulate cytoplasmic precursor, cells were exposed for the last 30 min of growth to 100 μg/ml bacitracin (see "Experimental Procedures").

* Peaks 3 and 4 were integrated together
the result of the loss of HCOOH and H₂O from the peak 258.2, as was shown before (38). Taken together, the MS/MS data provide a rigorous proof that the UDP-pentapeptide isolated from the COLVA strain of *S. aureus* grown in the presence of vancomycin contains D-lactate instead of D-alanine as the C-terminal residue of the cell wall precursor molecule.

**Fig. 5. Identification of the chemical structure of the UDPMurNAc-linked depsipeptide by MS/MS analysis.** Peak 8 of Fig. 4 was purified and analyzed by MS/MS. Steps in the fragmentation of the molecular ion at *m/z* 1151.6 are described in detail in the text. Structural assignments for the fragment ions detected are shown in C. Note that the masses of the observed fragment ions (numbers above arrows) are 1 Da greater than the masses of the neutral structures due to the addition of H⁺.

Inhibition of the Expression of Vancomycin Resistance by Sub-MIC Concentrations of Oxacillin—An overnight culture of COLVA grown in antibiotic-free TSB was plated at various dilutions on two sets of agar plates: one set contained only increasing concentrations of vancomycin in the range of 0.75 μg/ml to 1000 μg/ml. In the second set of vancomycin plates,
the agar medium also contained oxacillin at a constant concentration of 40 μg/ml. The number of bacterial colonies was determined after incubation at 37 °C for 48 h. All cells grew on the agar plates containing 250 μg/ml vancomycin, and the number of colonies dropped sharply on the plates containing 512 μg/ml vancomycin. The presence of oxacillin reduced the vancomycin MIC value from 512 to 12 μg/ml in 99.9% of the bacteria and generated a heterogeneous population profile (Fig. 6A).

Inhibition of the Expression of Oxacillin Resistance by Sub-MIC Concentrations of Vancomycin—An experiment similar to the one illustrated in Fig. 6A was performed to test the effect of sub-MIC concentrations of vancomycin on the oxacillin resistance of strain COLVA. Inclusion of 50 μg/ml vancomycin in the oxacillin plates reduced the oxacillin MIC of strain COLVA from 800 to 10 μg/ml in 99.9% of the bacteria, and the culture became heterogeneous in the oxacinillin-resistant phenotype (Fig. 6B).

Effect of Inactivation of mecA on Vancomycin Resistance and Cell Wall Composition of Strain COLVA—The Tn551-inactivated mecA (39) was transduced into strain COLVA to generate mutant COLVA-ΔmecA. The results of E-test showed that COLVA-ΔmecA has retained unaltered high level resistance to vancomycin but resistance to oxacillin was reduced to 10 μg/ml (Fig. 1B). Strain COLVA-ΔmecA was grown in TSB containing 250 μg/ml vancomycin, and cell walls and precursor pool prepared and analyzed as described. The cell wall precursor pool showed a composition very similar to that of COLVA grown in vancomycin-containing medium (Table III). The composition of the cell wall peptidoglycan is shown in Table II. It is apparent that COLVA with an inactivated mecA produced a muropeptide HPLC profile that was very similar to the muropeptide profile of strain COLVA that carried an intact mecA.

DISCUSSION

β-Lactam antibiotics are enzyme inhibitors and their mechanism of action involves inhibition of bacterial transpeptidases (penicillin-binding proteins, PBPs) that catalyze cell wall assembly. In contrast, glycopeptide antibiotics like vancomycin bind to the C-terminal of the cell wall precursor pentapeptide (Lipid II) and prevent it from being utilized for cell wall synthesis. The mechanisms of β-lactam and glycopeptide resistance match the mode of action of these antibiotics: β-lactam-resistant bacteria produce a surrogate transpeptidase with low
affinity for β-lactams (7–9). Vancomycin-resistant enterococci contain a cell wall precursor with altered C-terminal residue (depsipeptide), the bactoprenyl derivative of which (Lipid II) is compared to the normal PBPs of S. aureus (see wavy lines). The penicillin-binding protein 2A is unable to handle these D-lactate-containing precursors, which are polymerized and incorporated into the cell wall peptidoglycan by some of the normal PBPs of S. aureus.

The results of E-tests and population analysis clearly demonstrate that strain COLVA carrying both mecA and vanA gene complexes are expressed in the same staphylococcal cell.

The model for the synthesis of peptidoglycan in strain COLVA with or without an induction of the vanA operon. The UDP-MurNAc-pentapeptide cell wall precursor undergoes modifications: UDP is replaced by the undercarboxyprenylphosphate carrier lipid (wavy lines), catalyzed by MraY, followed by the attachment of N-acetylglucosamine (G) catalyzed by MurG to form Lipid II. After attachment of pentaglycine branches (FemX, FemA, and FemB), Lipid II is translocated to the outer surface of the plasma membrane where PBPs utilize it for cell wall assembly. Top panel: In the absence of the expression of vanA gene complex, strain COLVA produces a peptidoglycan built primarily of the normal UDP-MurNAc-pentapeptide cell wall precursors. In the presence of oxacillin, the penicillin-binding protein responsible for the polymerization of cell wall is PBP2A and the peptidoglycan contain muropeptides that carry the C-terminal d-alanyl-d-alanine residues. Lower panel: Upon expression of the vanA gene complex, the normal pentapeptide precursor is replaced by the n-lactate (white circle)-containing depsipeptide. Derivatization of this precursor by FemX, A, and B is incomplete resulting in Lipid II molecules defective in oligoglycine branches (black circle). The penicillin-binding protein 2A is unable to handle these n-lactate-containing precursors, which are polymerized and incorporated into the cell wall peptidoglycan by some of the normal PBPs of S. aureus. The peptidoglycan produced under these conditions contains only tetrapeptides in both monomeric and oligomeric muropeptide species. Symbols: N-acetylmuramic acid (M); N-acetylglycosamine (G); carrier lipid (wavy lines); regular stempeptide residues (black circles); n-lactate residue (white circles); glycine (gray circles). Open ellipsoid symbols: functionally inactive PBPs; solid ellipsoid symbols: functionally active PBPs.

A closer comparison of the muropeptide profile of strains COL and COLVA reveals several major differences. The HPLC profile of COLVA grown in drug-free TSB shows a number of minor peaks that are absent from strain COL grown under the same conditions. These minor peaks become the dominant muropeptide species in the peptidoglycan of COLVA grown in the presence of vancomycin. Analysis of the new muropeptide species by mass spectrometry showed that they lacked the fifth alanine residue from both the monomeric and also from the cross-linked muropeptide species; they were also deficient in the pentaglycine side chains, which are the dominant feature of the cell wall of COL and other S. aureus strains. The structures drawn in Fig. 3 suggest that the deficit in the pentaglycine branches was particularly frequent among monomers (see structures 1v through 9v in Fig. 3) and among muropeptides that may have served as the biosynthetically “first” donors during the formation of muropeptide dimers, trimers, and oligomers (see structures 11v, 12v, and 14v in Fig. 3). Yet another type of structural abnormality not observed before in strain COL was the occasional lack of amidation of the stem peptide β-glutamic acid residues (4v or 6va); the excess of 42 molecular weight units that we interpreted as the possible presence of an O-acetyl group (6va) and suggestive evidence for deacetylated hexosamines (16va and 17va).

Although strain COLVA was clearly capable of expressing...
high level resistance to both oxacillin and vancomycin, these two inhibitors had strong and mutual antagonist affects on the expression of the drug-resistant phenotypes. As little as 40 μg/ml oxacillin reduced the vancomycin MIC value of strain COLVA from 512 to 12 μg/ml. Similarly, as little as 50 μg/ml vancomycin reduced the oxacillin MIC of COLVA from 800 to 10 μg/ml. In both cases the homogeneous phenotypes were also converted to heterogeneous ones (Fig. 6).

The experiments illustrated in Figs. 6 and 1 provide important insights into differences in the mechanism of cell wall synthesis in bacteria expressing either the β-lactam or the vancomycin resistance genes. It has been shown that in strain COL the assembly of peptidoglycan in the presence of oxacillin is catalyzed by two PBPs: the transpeptidase activity of PBP2A and the transglycosylase activity of PBP2 (14, 15). The inhibition of expression of vancomycin resistance by oxacillin in strain COLVA suggests that the low affinity PBP2A, which is the only transpeptidase that remains active in the presence of oxacillin (13), is not able to utilize the depsipentapeptide wall precursors. The inhibition of vancomycin resistance by penicillin in enterococci has been interpreted in a similar manner. It was proposed that PBP5, which has low affinity to β-lactam, is unable to utilize depsipeptide wall precursors (43). Thus, the polymerization of UDP-MurNAc-depsipeptide precursor and its cross-linking in the peptidoglycan of strain COLVA must be catalyzed by one of the highly oxacillin sensitive native PBPs (Fig. 7). This interpretation is consistent with the composition of peptidoglycan of COLVA grown in the presence of oxacillin: except for the monomeric muropeptide 3v, all the other muropeptides were the “normal” structures characteristic of S. aureus grown in the presence of transpeptidase inhibitors (13). An direct experimental evidence for this interpretation came from the insertional inactivation of mecA in strain COLVA, which had no affect on the vancomycin MIC while it completely inactivated resistance to oxacillin (Fig. 1).

The sensitivity of the oxacillin-resistant phenotype to sub-MIC concentrations of vancomycin may be explained by the same mechanism. Induction of the vanA gene complex by vancomycin would lead to the destruction of the normal pentapeptide cell wall precursor (peak 6 in Fig. 4) and would flood the cytoplasmic cell wall precursor pool with the abnormal depsipeptide (peak 8 in Fig. 4). This abnormal cell wall precursor appears to be an inadequate substrate for PBP2A, which is the only transpeptidase available for wall synthesis in the presence of oxacillin in the medium. The inability of PBP2A to utilize depsipeptide precursors is reminiscent of early observations that documented the high degree of sensitivity of this protein to modification of the structure of the C termini of cell wall precursors (44, 45).

Our findings indicate that the vanA gene complex can be fully expressed in S. aureus with several features of the mechanism that were already identified in vancomycin-resistant enterococci (46). The novel aspect of our study was that we could follow the vanA-induced changes in cell wall metabolism from the composition of wall precursor pool all the way to the composition of the peptidoglycan. Earlier studies found no difference in the composition of the cell walls of vancomycin-resistant F. enterica (47, 48). However, a comparison of the cell wall structure of vancomycin-susceptible and vancomycin-resistant F. enterica has noted the complete absence of pentapeptide components (either β-alanine or β-lactate) from the peptidoglycan of the resistant bacteria (38, 49). It was proposed (49) that the β-lactate terminating acceptor peptides may be substrates of the decarboxypeptidase of which would remove the C-terminal β-lactate residues from the peptidoglycan of the resistant cells.

The complete replacement of pentapeptides by tetrapeptides in the peptidoglycan of COLVA grown in vancomycin-containing medium may be explained by a similar mechanism. However, the simultaneous presence of both normal (pentapeptide containing) and abnormal tetrapeptide-containing muropeptides in COLVA grown in antibiotic-free medium (Fig. 2B) implies that the enzyme involved must have a high degree of selectivity for the removal of terminal β-lactate acid residues.

The frequent absence or less than complete pentaglycine branch in muropeptide monomers of bacteria grown in the presence of vancomycin suggests that the depsipeptide containing Lipid II is not an ideal substrate for the FemX, -A, and -B proteins (50). Our data indicate that such abnormal depsipeptides carrying incomplete pentaglycine branches may nevertheless move forward in the biosynthetic pathway and incorporate preferentially as muropeptide monomers into the peptidoglycan. Such incomplete pentaglycine branches are also found in oligomeric components as the biosynthetically “first” donor muropeptides. This observation suggests that muropeptides defective in pentaglycine branches are not favored as acceptors in the transpeptidation reaction.

It was shown before that the sortase-dependent attachment of proteins occurs preferentially to pentaglycine branches in the cell wall of S. aureus (51). It remains to be seen if the deficit in pentaglycine branches in vancomycin resistant S. aureus also reduces attachment of surface-exposed proteins and the pathogenic potential of such strains.

The undiminished vancomycin resistance of the mutant strain of COLVA in which mecA was insertionally inactivated shows clearly that PBP2A, the key protein of β-lactam resistance, does not participate in the expression of vancomycin resistance but is dedicated to the biosynthesis of cell walls in the presence of β-lactam antibiotics. Polymerization of the depsipeptide-containing muropeptide must be catalyzed by some of the native PBPs of S. aureus. The two major antibiotic resistance mechanisms encoded by mecA and vanA residing in the same S. aureus appear to use different sets of enzymes for the assembly of cell walls.

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High Level Oxacillin and Vancomycin Resistance and Altered Cell Wall Composition in *Staphylococcus aureus* Carrying the Staphylococcal *mecA* and the Enterococcal *vanA* Gene Complex

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