Increased Production of Penicillin-Binding Protein 2, Increased Detection of Other Penicillin-Binding Proteins, and Decreased Coagulase Activity Associated with Glycopeptide Resistance in *Staphylococcus aureus*

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The mechanism of glycopeptide resistance in the genus *Staphylococcus* is unknown. Since these antimicrobial compounds act by binding the peptidoglycan precursor terminus, the target of transglycosylase and transpeptidase enzymes, it was hypothesized that resistance might be mediated in *Staphylococcus aureus* by increased production or activity of these enzymes, commonly called penicillin-binding proteins (PBPs). To evaluate this possibility, glycopeptide-resistant mutants were prepared by passage of several clinical isolates of this species in nutrient broth containing successively increasing concentrations of the glycopeptide vancomycin or teicoplanin. Decreased coagulase activity and increased resistance to lysostaphin were uniformly present in the vancomycin-resistant mutants. Peptidoglycan cross-linking increased in one resistant isolate and decreased in two resistant isolates. The amounts of radioactive penicillin that bound to each PBP in susceptible and resistant strains were compared; PBP2 production was also evaluated by Western blotting. Increased penicillin labeling and production of PBP2 were found in all resistant derivatives selected by either vancomycin or teicoplanin. Moreover, the increase in PBP2 penicillin labeling occurred early in a series of vancomycin-selected derivatives and was strongly correlated ($r > 0.9$) with the increase in vancomycin and teicoplanin MIC. An increase in penicillin labeling also occurred, variably, in PBP1, PBP3, and/or PBP4. These data demonstrate a strong correlation between resistance to glycopeptides and increased PBP activity and/or production in *S. aureus*. Such an increase could allow PBPs to better compete with glycopeptides for the peptidoglycan precursor.

Until recently, the glycopeptide vancomycin was the only licensed antibacterial compound to which methicillin-resistant *Staphylococcus aureus* clinical isolates have remained uniformly susceptible (25). However, the identification of *S. aureus* clinical isolates with intermediate resistance to vancomycin (MIC, 8 μg/ml) (9) and another glycopeptide, teicoplanin (MIC, 8 to 16 μg/ml) (10, 15), raises the possibility that resistance to glycopeptides in *S. aureus* will soon become an important clinical problem. To provide a model for studying mechanisms of glycopeptide resistance in *S. aureus*, we (6) have selected mutants with decreased susceptibility (resistance) to glycopeptides by incubating clinical isolates in vancomycin or teicoplanin.

For two such glycopeptide-resistant mutants prepared in our laboratory by incubation in vancomycin, the MIC of both vancomycin and teicoplanin was increased (6). Additionally, observed in the resistant mutants were several alterations in phenotype, including smaller colony size, lower growth rate, larger cell diameter, thicker cell walls, decreased susceptibility to lysostaphin (an enzyme that cleaves staphylococcal peptidogly-

can in the pentaglycine cross-bridges), decreased zone sizes of beta-hemolysis, and loss of phage and capsular type (6). In addition, one of these resistant mutants overproduced a D-lactate dehydrogenase (16) similar in structure and function to the VanH dehydrogenase essential for glycopeptide resistance in *Enterococcus faecium* and *Enterococcus faecalis* (1). However, insertional inactivation of the *ddh* gene encoding the dehydrogenase in *S. aureus* did not alter the glycopeptide resistance phenotype (3). Therefore, the mechanism of vancomycin resistance in laboratory-derived glycopeptide-resistant *S. aureus* isolates is distinct from the mechanism used by enterococci and remains uncertain.

An alternative hypothesis to explain glycopeptide resistance in *S. aureus* can be inferred from the mechanism of action of these antibiotics which interfere with peptidoglycan synthesis. Such interference is accomplished by the drug binding to the D-Ala-D-Ala termini of the stem pentapeptide portion of peptidoglycan precursors, thereby sterically blocking transglycosylation and transpeptidation reactions (2). Since transpeptidases are likely to bind to the same D-Ala-D-Ala termini of peptidoglycan precursors (12) to which glycopeptides bind, resistance to these agents could result from overproduction of or alterations in transpeptidases that increase their affinity for D-Ala-D-Ala.

The transpeptidases and carboxypeptidases covalently bind penicillin and, as such, are commonly referred to as penicillin-binding proteins (PBPs) (13, 23). By use of a penicillin binding assay, four PBPs (PBP1 to -4) can be detected in *S. aureus* with molecular masses that range between 85 and 45 kDa (13). In

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TABLE 1. *S. aureus* isolates and relevant MIC data

Isolate[a]	Source	GP[b]	Selecting concn (μg/ml)[c]	MIC (μg/ml) of agent[d]		Coagulase detection time (h)	Reference or source
				Vm	Tco		
523	Human hip	Vm	NA	1	2	1.5	6
523a	523	Vm	1	3	3	ND[e]	6
523c	523	Vm	3	4	8	ND	6
523k	523	Vm	7.5	8	8	24	6
523$_{teico}$	523	Tco	130	4	128	3	This study, same as 523v5
1714	Human blood	Vm	NA	2	2	1.5	This study
1714s	1714	Vm	27	32	8	4	This study
1715	Human blood	Vm	NA	2	1	1.5	This study
1715w	1715	Vm	25	16	16	24	This study

[a] The lowercase letters indicate the step of isolation in the serial incubation protocol which is described in Materials and Methods. 523a, -c, -g, and -k are serial isolates derived by incubation of isolate 523 in vancomycin. 523$_{teico}$ (isolate 523v5) is the only terminal isolate derived by incubation in teicoplanin. All other resistant isolates, 523k, 1714s, and 1715w, are terminal isolates derived by incubation in vancomycin.

[b] GP, the glycopeptide used for selection: Vm, vancomycin; Tco, teicoplanin.

[c] Concentration of glycopeptide in which isolate was selected. NA, not applicable (parent isolate).

[d] In determining the MICs for isolates 1714s and 1715w, wells containing clumps of bacteria or turbidity were interpreted as having growth. For isolates 523a, -c, -g, and -k, MIC determinations were performed with drug concentrations of 0, 1, 2, 3, 4, and 8 μg/ml. All other MICs were determined with the use of twofold serial dilutions of the agent; the lowest drug concentration tested was 1 μg/ml.

[e] ND, not determined.

an *S. aureus* clinical isolate with low-level teicoplanin resistance (22), an increase in penicillin labeling of PBP2 was found compared with that in an epidemiologically related susceptible isolate. However, whether such an increase also occurs in the PBPs of vancomycin-resistant *S. aureus* isolates has not been previously investigated.

Our aim was to produce laboratory-derived resistant isolates from various parent clinical strains by incubation in either vancomycin or teicoplanin in order to identify common properties among glycopeptide-resistant *S. aureus* strains. We found increased penicillin PBP2 labeling in all the vancomycin- and teicoplanin-resistant mutants tested compared with the parent strains. Since increased penicillin labeling could reflect increased PBP2 production or increased affinity of this transpeptidase for penicillin, an analog of the D-Ala-D-Ala terminus of the peptidoglycan precursor (24), PBP2 production was assessed by Western blotting.

MATERIALS AND METHODS

Bacterial isolates and procedure for selecting glycopeptide-resistant clones. Techniques used for identification of *S. aureus* isolates and conditions for growth and storage were previously described (6). Relevant information regarding glycopeptide-resistant derivatives and susceptible clinical parent strains is summarized in Table 1. Isolation of glycopeptide-resistant derivatives from clinical isolates was performed as described previously (6) by serial incubation in Trypticase soy broth containing increasing concentrations of either vancomycin or teicoplanin until the highest concentration of drug that could select a resistant subpopulation was reached. The isolates are referred to by the parent strain designation and a letter (a, b, c, etc.) (Table 1) indicating the succession of their isolation from the clinical parent. For example, isolate 523k was the 11th derivative in one series of incubations of isolate 523. The resistant derivative which was obtained from the highest glycopeptide concentration is referred to as the terminal derivative. Isolate 523v5 is a terminal derivative obtained by incubation of isolate 523 in teicoplanin and is therefore referred to in the text as 523$_{teico}$ to clearly distinguish it from all other resistant terminal isolates shown in Table 1, which were obtained by incubation in vancomycin.

The glycopeptide-resistant series 523a to -k, obtained from the β-lactamase-producing clinical isolate 523 during serial incubation in vancomycin, was previously described (6). Isolate 523k, like its parent isolate 523, produces β-lactamase. Since differences in penicillin binding activity between 523k and 523 could be due to possible differences in β-lactamase activity between the isolates, by causing different rates of hydrolysis of the radiolabeled penicillin used in the penicillin binding assay, two isolates (1714 and 1715) that do not elaborate β-lactamase were obtained from the blood of adult patients and used as parent strains to derive additional vancomycin-resistant lineages. Confirmation that the terminal resistant isolates were derived from the wild-type susceptible isolates was obtained by pulsed-field gel electrophoresis.

MIC determination, antimicrobial agents, β-lactamase, lysostaphin digestion, and coagulase tests. The MIC of vancomycin and teicoplanin was determined by broth microdilution with the use of Ca^{2+}- and Mg^{2+} (cation)-supplemented Mueller-Hinton broth according to the guidelines of the National Committee for Clinical Laboratory Standards (18). Unless indicated otherwise, twofold serial dilutions of drug were used; the lowest concentration evaluated was 1 μg/ml. Vancomycin was obtained from Sigma, St. Louis, Mo. Clavulanic acid and teicoplanin were gifts from SmithKline Beecham, Philadelphia, Pa., and Marion Merrell Dow Inc., Cincinnati, Ohio, respectively.

β-Lactamase production was detected by the color change occurring with nitrocefin hydrolysis (19). For comparison of lysostaphin susceptibility, lysostaphin digestion was performed on whole cells as previously described (6). Tube coagulase tests of parent and resistant derivatives were performed with similar inocula (~10^{10} CFU) with the same lot of rabbit plasma (Difco, Detroit, Mich.) according to the recommendations of the manufacturer. The assay mixture was incubated in a 37°C water bath for 4 h and then at room temperature overnight. Tubes were inspected for clot formation at 30-min intervals until 6 h and, subsequently, at 11 and 24 h.

Analysis of PBP profiles by penicillin binding assay. (i) Preparation of membrane fractions. Cells grown to logarithmic phase (~5×10^8 CFU/ml) were harvested by centrifugation at 12,210 × *g* and washed with 50 mM phosphate buffer (pH 7.0). To disrupt cell walls, cells were incubated in 50 mM phosphate buffer (pH 7.8) containing lysostaphin (Sigma) (250 to 500 μg/ml) and DNase (Sigma) (10 μg/ml) at 37°C for 20 to 60 min. To decrease the viscosity of the lysostaphin-treated suspension, sonication was performed for 30 s as described previously (6); whole cells and cellular debris were removed by centrifugation at 12,000 × *g* for 15 min. Membrane fractions were obtained by centrifugation at 208,000 × *g* for 45 min at 4°C, resuspension in 50 mM phosphate buffer (pH 7.2), and sonication for 3 s to resuspend the pellet. Fractions were stored at −70°C.

(ii) Detection of PBPs. Protein concentrations were determined by the method of Bradford (4) with the use of the Bio-Rad protein assay kit (Hercules, Calif.). Membrane proteins (50 to 100 μg) were incubated in a final volume of 75 μl with 10 to 50 μl of [^{14}C]benzylpenicillin (158 μCi/mg, 50 μCi/ml) or [^{3}H]benzylpenicillin (1.0 mCi/ml, 10 to 30 Ci/mmol) (Amersham, Arlington Heights, Ill.) for 10 min at 37°C. The reaction was terminated by addition of Laemmli buffer (14) and boiling for 3 min. The entire reaction mixture was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14) as described previously (6) in a 4% polyacrylamide stacking gel and a 10% separating gel 16 cm in length. Molecular weight standards (Bio-Rad) were run in parallel to the samples. Electrophoresis was performed in a Protean II slab cell apparatus (Bio-Rad) at 100 V. To allow clear separation of the PBP bands, electrophoresis was performed until the 29-kDa prestained marker (Bio-Rad) approached the bottom of the gel. Gels were stained with 0.1% Coomassie brilliant blue, destained, incubated with fluor (Amplify; Amersham) for 30 min, and dried overnight at 37°C on cellophane (Hoefer Scientific Instruments, San Francisco, Calif.) in a Tut's Tomb gel drying frame (Research Products International, Mount Prospect, Ill.). Dried gels were exposed to Hyperfilm (Amersham) for 4 weeks after labeling with [^{14}C]benzylpenicillin or for 4 to 37 days after labeling with [^{3}H]benzylpenicillin.

Densitometry and statistical methods. Films and gels were scanned with an OmniMedia flatbed optical scanner, Model 12 cx (Lumisys, Sunnyvale, Calif.). Densitometric analyses on scanned images were performed with the use of the

public domain NIH Image software (version 1.57) by measuring the area under the curve produced by each signal. Arbitrary density units were converted to optical density by calibration with an optical density step tablet (Kodak). The optical density of the bands on fluorograms or Western blots was corrected for protein by determining the ratio between the optical density of the band and the optical density of the sum of all the bands measured on the Coomassie blue-stained gel. Correlation between corrected PBP band intensities and the vancomycin MIC for the corresponding isolate was determined by linear regression analysis performed with Cricket Graph software (version 1.10).

Analysis of PBP2 by Western blotting. Membrane fractions containing 12.5 μg of protein from each isolate were separated by SDS-PAGE and electroblotted onto nitrocellulose (Bio-Rad) in a Trans-Blot semidry transfer apparatus (Bio-Rad) as recommended by the manufacturer. Nitrocellulose membranes were blocked in skim milk and incubated with polyclonal rat anti-PBP2 antiserum (dilution of 5×10^{-4}), kindly provided by Kazuhisa Murakami (Shionogi Research Laboratories, Osaka, Japan) (17). The secondary antibody (alkaline phosphatase-conjugated goat anti-rat immunoglobulin G) (Sigma) was used at a dilution of 2.5×10^{-3} and detected by incubation with BCIP (5-bromo-4-chloro-3-indolyl phosphate) (Sigma) and nitroblue tetrazolium (Sigma) according to the manufacturer's instructions.

Muropeptide analysis of peptidoglycan by reverse-phase high-performance liquid chromatography (RP-HPLC). Peptidoglycan was obtained from cell walls of susceptible and resistant isolates grown to mid-exponential phase as described elsewhere (7). Teichoic acid was removed from cell walls by hydrofluoric acid treatment, and peptidoglycan was hydrolyzed with muramidase isolated from *Streptomyces globilosporus* (Sigma). The muropeptide products were reduced with sodium borohydride and separated by RP-HPLC with the use of a linear methanol gradient (5 to 30%) in 100 mM sodium phosphate buffer (pH 2.5). Muropeptide standards (7) were used to identify the peaks. The structure of muropeptides of interest was determined by fast atom bombardment-mass spectrometric analysis (7).

RESULTS

Isolation and characterization of new glycopeptide-resistant mutants. To study vancomycin resistance mechanisms in diverse genetic backgrounds, the β-lactamase-negative clinical isolates 1714 and 1715 were incubated in stepwise increasing vancomycin concentrations to obtain terminal resistant isolates 1714s and 1715w, respectively (Table 1). A terminal isolate was produced also by selecting strain 523 with teicoplanin and was called 523v5 or 523_{teico}. Notably, this isolate was selected from a much higher concentration of drug than were any of the isolates selected in vancomycin (130 μg of teicoplanin per ml versus 27 μg of vancomycin per ml) (Table 1).

The resistant isolates 1714s and 1715w usually formed clumps in liquid medium, a tendency which increased in the presence of glycopeptide. As shown in Table 1, all terminal isolates selected in vancomycin had low-level resistance to vancomycin and low-level cross-resistance to teicoplanin. Isolate 523_{teico} had a 64-fold increase in the MIC of teicoplanin (MIC, 128), which far exceeded the teicoplanin MIC for any terminal isolate produced when vancomycin was the selecting agent (Table 1). 523_{teico} had cross-resistance to vancomycin (Table 1), but the vancomycin MIC change was relatively minimal.

In addition to the MIC changes, several consistent differences in phenotype were observed between the terminal resistant derivatives (1714s, 1715w, and 523_{teico}) and the respective, susceptible parent isolate (Table 1). These changes were similar to those previously observed between 523k and 523 (6). First, the resistant mutants were less susceptible to lysis by lysostaphin. One-hour incubations with this enzyme were necessary to decrease the turbidity of suspensions of the resistant isolates evaluated, compared with glycopeptide-susceptible isolates, which required only 20 min. Second, in the tube coagulase test, an extended time was required for clot formation by the resistant derivatives compared with the respective parent strains (Table 1). Third, the resistant derivatives grew more slowly in liquid broth and produced smaller colonies on solid medium than the respective parent strain.

Increased detection of PBPs in all glycopeptide-resistant isolates. As shown in the fluorogram from a representative
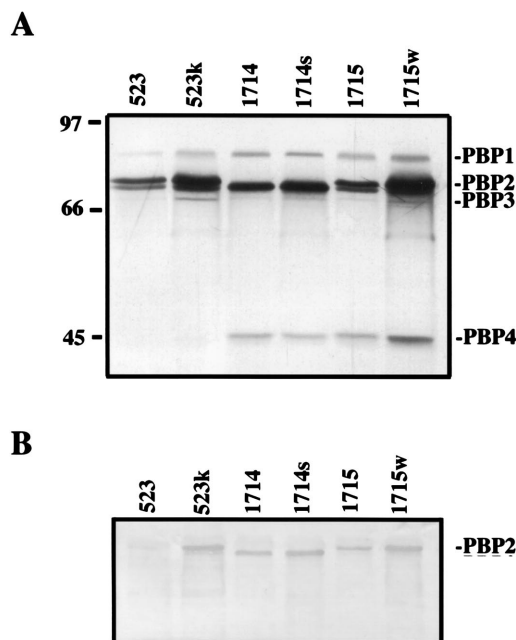

FIG. 1. Increased detection of PBPs in vancomycin-resistant *S. aureus* isolates. (A) Fluorogram of membrane proteins (100 μg) incubated with 50 μl (7.5-μg/ml final concentration) of [³H]penicillin and separated by SDS-PAGE. X-ray film was exposed to the dried gel for 20 days. Numbers at left are molecular masses in kilodaltons. (B) Western blot of membrane fractions probed with PBP2 antiserum. Images were produced by optical scanning and were lettered with the use of Adobe Photoshop software (version 2.5.1) (Adobe Systems, Inc.).

penicillin binding assay (Fig. 1A), the signal for PBP2 was increased in the resistant isolates 523k (3.6-fold), 1714s (2.5-fold), and 1715w (6.1-fold) compared with the respective parent isolates. The presence of two closely migrating bands detected by penicillin labeling in isolates 523, 523k, 1715, and 1715w is consistent with the presence of the two bands of similar molecular mass previously reported for the PBP2 complex (5, 22). Thus, increases in PBP2 labeling were documented in vancomycin-resistant derivatives selected from all three genetic backgrounds evaluated. The intensity of the PBP2 signal (after correction for protein) from the three pairs of isogenic parent-terminal derivatives (1714-1714s, 1715-1715w, and 523-523k) evaluated on the same fluorogram strongly correlated with the vancomycin and teicoplanin MICs for these isolates ($r = 0.97$ and 0.95 for vancomycin and teicoplanin MICs, respectively).

In addition to the increases in PBP2, densitometric analysis of bands in Fig. 1A revealed an increase in at least one other PBP (PBP1, PBP3, and/or PBP4) for each resistant derivative compared with the respective susceptible parent derivatives. PBP1 was increased in isolates 523k, 1714s, and 1715w; PBP3 was increased in 1714s; and PBP4 was increased in 1715w. In Fig. 1A, PBP4 was detected in amounts in 523 and 523k insufficient to assess a difference between these isolates. However, in another film (data not shown) PBP4 was apparently increased in 523k since PBP4 could be detected by penicillin labeling in 523k but not in 523. Also, a penicillin-labeled band with a molecular mass of ca. 53 kDa, not detected in susceptible isolates, was increased in the resistant derivatives 523k, 1714s, and 1715w (Fig. 1A).

In isolate 523_{teico}, the penicillin PBP2 labeling increased 2.4-fold relative to that for 523 (Fig. 2A). This increase was similar in magnitude to that documented for the resistant iso-
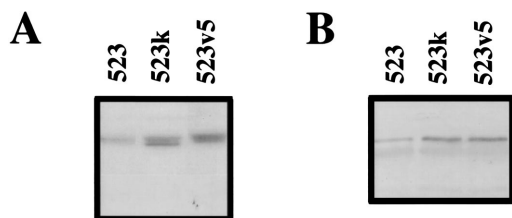
FIG. 2. Increased detection of PBP2 in a teicoplanin-resistant derivative of 523. (A) Fluorogram of membrane fractions (100 μg of each isolate) incubated with 10 μl (42-μg/ml final concentration) of [^{14}C]penicillin and separated by SDS-PAGE. Film was exposed to the gel for 46 days. (B) Western blot probed with anti-PBP2 antiserum. Images in the figure were produced by optical scanning and were lettered with the use of Adobe Photoshop software (version 2.5.1) (Adobe Systems, Inc.).
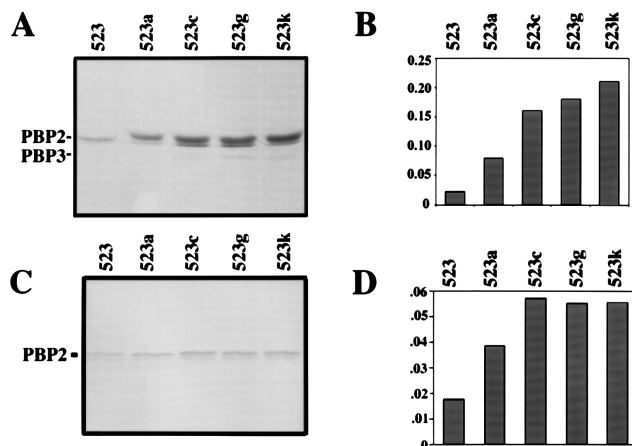

FIG. 3. Increased penicillin labeling and production of PBP2 in the series of vancomycin-resistant isolates derived from isolate 523. (A) Fluorogram of membrane proteins (50 μg) incubated with 10 μl (1.5-μg/ml final concentration) of [^{3}H]penicillin and separated by SDS-PAGE. X-ray film was exposed to the dried gel for 37 days. (B) Optical density of the PBP2 signal in the film shown in panel A corrected for optical density of the protein contained in the corresponding lane of the SDS-PAGE gel. (C) Western blot of membrane fractions from indicated isolates probed with PBP2 antiserum. (D) Optical density of the signal for PBP2 in the Western blot shown in panel C corrected for optical density of the protein in the corresponding lane of the SDS-PAGE gel. Images in panels A and C were produced by optical scanning and were lettered with the use of Adobe Photoshop software (version 2.5.1).

lates selected in vancomycin, which had low-level teicoplanin resistance.

The Western blots of membrane fractions of all tested resistant isolates, 523k, 1714s, 1715w (Fig. 1B), and 523$_{teico}$ (Fig. 2B), showed increased amounts of a band (or bands) similar in molecular mass to PBP2 compared with the susceptible parent. The fold increases were 1.8, 5.6, 1.7, and 2 in 523k, 1714s, 1715w (Fig. 1B), and 523$_{teico}$ (Fig. 2B), respectively. Similar to the finding in the penicillin binding assay, a faint band with a molecular mass of 53 kDa (did not reproduce well on the scanned image) not characteristic of known PBPs of *S. aureus* was detected by the antiserum in the resistant isolates 523k, 1714s, and 1715w (Fig. 2B). The reactivity with the anti-PBP2 antiserum indicated that the band could be a proteolytic product of PBP2 not detected when PBP2 is produced at wild-type levels.

Detection of PBP2 in a series of isolates derived from 523. In penicillin binding assays of the serial isolates obtained in the same series as 523k, the increased signal of the bands corresponding to the PBP2 complex was evident in the first isolate in the series (523a, Fig. 3A and B) and continued; a further increase occurred in each subsequent isolate. The largest incremental increases in the PBP2 signal between isolates in the series were between isolates 523 and 523a and between 523a and 523c. The mean fold increases relative to strain 523 in the PBP2 signal in 523a, 523c, 523g, and 523k were 2.3, 5.5, 6.9, and 8.2, respectively. The intensity of the PBP2 signal (Fig. 3A and B) strongly correlated with the vancomycin MIC for these isolates ($r = 0.95$).

In Western blots of serial isolates 523a, 523c, 523g, and 523k (Fig. 3C), increases of 2.2- and 3.2-fold were observed (Fig. 3D) in 523a and 523c, respectively, compared with 523. The signal for PBP2 in isolates 523g and 523k was similar in magnitude to that of 523c. Thus, stepwise increases occurred in PBP2 production in serial isolates 523a and 523c, with no further increases occurring in subsequent isolates. This pattern was similar to the results obtained for these isolates in the penicillin binding assay (Fig. 3A and B), in which most of the increase in PBP2 occurred in the early isolates 523a and 523c.

Analysis of peptidoglycan composition in susceptible and resistant isolates. Since PBP1, PBP2, and PBP3 are believed to catalyze transpeptidation reactions that cross-link peptidoglycan (13), we investigated by RP-HPLC analysis whether peptidoglycan composition and cross-linkage are affected by increased PBP production or activity in three of the resistant isolates.

Except for the unresolved material eluting after ca. 110 min in the chromatogram (i.e., highly cross-linked muropeptides [Fig. 4]), the peptidoglycan composition of the resistant and

susceptible isolates was very similar. The relative abundance of the unresolved material in the resistant isolate varied among the sets of strains. Whereas increased polymerization of peptidoglycan was observed in 523k compared with strain 523 (Fig. 4A and B), decreased polymerization was observed in 1715w compared with 1715 (Fig. 4C and D) and between 1714s and 1714 (data not shown). These data suggest that there is no consistent association between degree of cross-linking in pep-
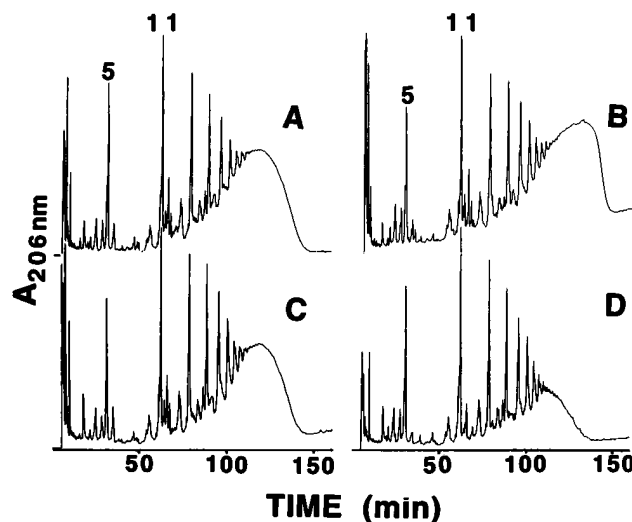

FIG. 4. HPLC profiles of muropeptides from susceptible and vancomycin-resistant isolates. (A) 523; (B) 523k; (C) 1715; (D) 1715w. Muropeptide peaks of *S. aureus* were previously analyzed and identified by mass spectroscopy and amino acid composition (7). The structures of peaks 5 and 11 in isolates 523 and 523k contain pentaglycine-substituted D-Ala-D-Ala-terminating muropeptide monomer and dimer, respectively, as determined by mass spectroscopy.

tidoglycan and either PBP production or vancomycin resistance.

Analysis of peaks appearing prior to ca. 110 min revealed no novel muropeptide species in the vancomycin-resistant isolates. The major monomeric and dimeric compounds of the strains (peaks 5 and 11) consisted of pentaglycine-substituted D-Ala-D-Ala-terminating muropeptides as judged by retention time on the RP-HPLC chromatogram (7). Moreover, for isolates 523 and 523k, the assumed structures of peak 5 and 11 were confirmed by mass spectrometry (data not shown).

DISCUSSION

In this report, we show a strong correlation between increased penicillin labeling of PBP2 and low-level vancomycin and teicoplanin resistance in laboratory-derived vancomycin-resistant *S. aureus* isolates. Such increased labeling by penicillin involves increased production of PBP2, since increases were detected also by Western blotting. Moreover, the increase in PBP2 penicillin labeling and production occurred in an early step of the serial incubation procedure in association with the first increase in the vancomycin MIC. These findings may be clinically relevant since we have found (unpublished data) similar high-level PBP2 production in intermediately vancomycin-resistant (MIC, 8 μg/ml) methicillin-resistant *S. aureus* clinical isolates from Japan which were obtained after vancomycin treatment failure (9). The level of vancomycin resistance in such isolates is similar to that of our laboratory-derived isolates.

There are several important differences between our findings and those of others in which increased PBP2 penicillin labeling was demonstrated in clinical *S. aureus* isolates with decreased susceptibility to teicoplanin (15, 22). First, our laboratory-derived mutants had decreased susceptibility to both vancomycin and teicoplanin, whereas the teicoplanin-resistant clinical isolates previously studied (15, 22) had no increase in the MIC of vancomycin. Second, by use of the Western blotting assay, we demonstrated that the increased PBP2 penicillin labeling documented in the laboratory-derived glycopeptide-resistant isolates involves an increase in PBP2 production. Third, our use of laboratory-derived resistant isolates enabled us to directly compare PBP production in resistant mutants with that in their related parent clinical isolates that were susceptible to glycopeptides. Obviously, such comparison is not always possible when clinical isolates are studied. Finally, no alterations in either PBP1, PBP3, or PBP4 were described in teicoplanin-resistant clinical isolates (15, 22), as was found in our isolates. The lack of increase in these other PBPs in the teicoplanin-resistant clinical isolates could be due to the absence of vancomycin resistance or the variability among different resistant isolates we found with respect to increases in these other PBPs.

These data suggest that PBP2, and perhaps other PBPs, may play a direct role in the development of vancomycin or teicoplanin resistance. Since vancomycin and a PBP are likely able to sterically block binding by the other to the D-Ala-D-Ala terminus of the peptidoglycan precursor (12), increased production of a PBP might increase the concentration of glycopeptide that is needed to interfere with the interaction between a PBP and the D-Ala-D-Ala substrate during peptidoglycan synthesis. The same effect could be realized by a change in PBP primary sequence that alters the affinity for D-Ala-D-Ala. Although we have not entirely excluded the latter possibility, increased production of PBP2 could explain most of the increase in penicillin labeling of this enzyme.

Alternatively, it is possible that PBPs do not play a direct

role in glycopeptide resistance. Since PBPs play an important role in bacterial replication, growth, and morphogenesis (8, 21), it is possible that PBP production or activity is increased in the resistant mutants in response to mutations that produce the characteristic slow-growth phenotype. Alternatively, expression of the genes encoding the PBPs may be coregulated along with that of genes encoding another protein(s) which is responsible for resistance.

The pleiotropic cell-surface-related changes that commonly occur in the glycopeptide-resistant mutants (e.g., coagulase activity and lysostaphin susceptibility) may reflect alterations in the cell surface which could decrease the efficiency with which vancomycin interacts with its target and could stabilize the resistance phenotype. In this regard, we did not document differences in the HPLC chromatograms of muropeptides from three pairs of related susceptible and vancomycin-resistant isolates except for a slight difference in cross-linking between susceptible and resistant isolates. Such differences in cross-linking could be related to the changes in activity caused by the differing amounts of PBPs in the separate isolates. However, the similar peptidoglycan structures we found in comparing vancomycin-resistant with susceptible *S. aureus* isolates indicate that the differences in lysostaphin sensitivity within these pairs cannot be explained by structural or compositional differences in peptidoglycan. It is possible that lysostaphin resistance is a result of increased peptidoglycan material comprising the thicker cell wall of resistant isolates, such as occurs in resistant isolate 523k (6). Such a thicker cell wall might act as a barrier to retard penetration of vancomycin through the cell wall. The increased time necessary for coagulase detection in glycopeptide-resistant derivatives was as consistent a change as the increase in PBP2 production. This observation may have important clinical implications, since coagulase production is often used to identify *S. aureus* (11). In this regard, the decrease in coagulase activity in vancomycin-resistant isolates could, on occasion, lead to inaccurate species-level identification of vancomycin-resistant *S. aureus* isolates if the coagulase tube test is not extended beyond 4 h.

Transglycosylase activity has not been detected in *S. aureus* PBPs (20). However, since glycopeptides interfere with transglycosylase as well as transpeptidase activities, increases in PBP2 in glycopeptide-resistant mutants might signify the presence of a previously undetected transglycosylase activity in PBP2 that could supplement the major transglycosylase when vancomycin is present. In support of this idea, a putative transglycosylase domain has been identified in the deduced N-terminal sequence of *S. aureus* PBP2 (17).

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