Inactivated \( \text{pbp4} \) in Highly Glycopeptide-resistant Laboratory Mutants of \textit{Staphylococcus aureus}*

(Received for publication, February 12, 1999, and in revised form, April 8, 1999)

Krzysztof Sieradzki, Mariana G. Pinho, and Alexander Tomasz‡

From The Rockefeller University, New York, New York 10021

Both vancomycin- and teicoplanin-resistant laboratory mutants of \textit{Staphylococcus aureus} produce peptidoglycans of altered composition in which the proportion of highly cross-linked mucopeptide species is drastically reduced with a parallel increase in the representation of mucopeptide monomers and dimers (Sieradzki, K., and Tomasz, A. (1997) \textit{J. Bacteriol.} 179, 2557–2566; and Sieradzki, K. and Tomasz, A. (1998) \textit{Microb. Drug Resist.} 4, 159–168). We now report that the distorted peptidoglycan composition is related to defects in penicillin-binding protein 4 (PBPs); no PBP4 was detectable by the fluorographic assay in membrane preparations from the mutants, and comparison of the sequence of \( \text{pbp4} \) amplified from the mutants indicated disruption of the gene by two types of abnormalities, a 17-amino acid long duplication starting at position 305 of the \( \text{pbp4} \) gene was detected in the vancomycin-resistant mutant, and a stop codon was found to be introduced into the \( \text{pbp4} \) KTG motif at position 281 in the mutant selected for teicoplanin resistance. Additional common patterns of disturbances in the peptidoglycan metabolism of the mutants are indicated by the increased sensitivity of mutant cell walls to the M1 muramidase and decreased sensitivity to lysostaphin, which is a reversal of the susceptibility pattern of the parental cell walls. Furthermore, the results of high performance liquid chromatography analysis of lysostaphin digests of peptidoglycan suggest an increase in the average chain length of the glycan strands in the peptidoglycan of the glycopeptide-resistant mutants. The increased molar proportion of mucopeptide monomers in the cell wall of the glycopeptide-resistant mutants should provide binding sites for the “capture” of vancomycin and teicoplanin molecules, which may be part of the mechanism of glycopeptide resistance in \textit{S. aureus}.

In an attempt to obtain some insights into the mechanism of staphylococcal glycopeptide resistance, we isolated laboratory step mutants using either vancomycin (6) or teicoplanin (7) as the primary selective agent. Despite several differences in the properties of mutants selected by vancomycin as compared with those selected by teicoplanin, both types of highly glycopeptide-resistant mutants showed decreased cross-linking of mucopeptides and several other properties, suggesting extensive perturbation of cell wall metabolism. In this communication we use a combination of biochemical and genetic techniques to further explore the mechanism that has led to the striking changes in cell wall structure and metabolism of these mutants.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—The parental strain for both of the independently isolated glycopeptide-resistant mutants was the methicillin-resistant \textit{S. aureus} strain COL (8). Mutant VM was isolated by serial selection with vancomycin (6) and mutant TM by selection for bacteria capable of growing on tryptic soy agar containing increasing concentrations of teicoplanin (final concentration, 100 \( \mu \text{g/ml} \)) (7). A third mutant, TM, was derived from mutant VM as a spontaneous, single step teicoplanin-resistant derivative, capable of growing on agar containing 800 \( \mu \text{g/ml} \) teicoplanin (6). The methicillin-susceptible strain RN450 (9) was also used in some of the experiments. The antibiotic susceptibility profiles of these strains are shown in Table I. All strains were grown in tryptic soy broth (Difco, Detroit, MI) at 37 °C with aeration. For each experiment, overnight cultures were diluted 10,000-fold into prewarmed tryptic soy broth and growth was followed by monitoring optical density (620 nm, using an LKB Spectrophotometer, Amersham Pharmacia Biotec, Sweden) and by plating on tryptic soy agar to determine viable titers of the cultures. Antibiotic resistance levels were determined by plating diluted cultures on tryptic soy agar for population analysis, as described previously (10).

**High Performance Liquid Chromatography (HPLC)**—Analysis of Peptidoglycan—Cell wall peptidoglycan was prepared and enzymatic cell wall hydrolases were analyzed with reversed-phase HPLC as described previously (11), except that the alkaline phosphatase step was omitted. Peptidoglycan prepared from the parental strain and from the three resistant mutants was analyzed after digestion with three different types of enzymes. In the first type of enzymatic hydrolysis, digestion by the M1 muramidase was used. In the second type of digestion, M1 was replaced by lysostaphin, and in the third type of digestion lysozyme treatment was followed by a second digestion with the M1 muramidase.

**Susceptibility of Cell Walls to Enzymatic Digestion in Vitro**—Purified cell walls were suspended in appropriate buffer (for lysostaphin, 50 mM Tris-Cl, pH 7.5; for muramidase, 25 mM phosphate buffer, pH 5.5) to initial \( A_{620} = 1.0 \). Lysis was measured as a decrease in \( A_{620} \) during incubation of the wall samples at 37 °C.

**Membrane Purification and Analysis of Penicillin-binding Proteins (PBPs)**—Membranes were prepared from cells grown to the late exponential stage in the following way: harvested cells were washed once in 50 mM Tris, 150 mM NaCl, 5 mM MgCl₂, pH 7.5, resuspended in the same buffer supplemented with phenylmethylsulfonyl fluoride (0.5 mM) and \( \beta \)-mercaptoethanol (10 mM). Lysostaphin, DNase, and RNase

* Partial support for these investigations was received from the Irene Diamond Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Supported by a grant from PRAXIS XXI/BD/9079/96.
§ To whom correspondence should be addressed: The Rockefeller University, 1230 York Ave., New York, NY 10021. Tel.: 212-327-8278; Fax: 212-327-8688.

---

*The abbreviations used are: HPLC, high performance liquid chromatography; PBP, penicillin-binding protein; PCR, polymerase chain reaction.
TABLE I
Antibiotic susceptibility profiles of Staphylococcus aureus strains used in the study

| Strain  | Methicillin | Vancomycin | Teicoplanin |
|---------|-------------|------------|-------------|
| COL     | 800         | 1.5        | 3.0         |
| VM      | 1.5         | 100        | 50          |
| TM      | 0.75        | 100        | >3,200      |
| TNM     | 200         | 6.0        | 200         |
| RN450   | 0.75        | 0.4        | 0.4         |

FIG. 1. HPLC elution profiles of muropeptides isolated from the parental strain COL, vancomycin-resistant mutant VM, vancomycin- and highly teicoplanin-resistant mutant TM, and teicoplanin-resistant mutant TNM. Peptidoglycan was purified and digested with muramidase (A), lysostaphin (B), and lysostaphin followed by muramidase (C). Separation of muropeptides was performed as described previously (11).

FIG. 2. Muropeptide composition of parental strain COL and its glycopeptide-resistant mutants VM, TM, and TNM. Percentages are expressed as the fraction of the total area and were obtained from HPLC profiles shown in Fig. 1. Muropeptide numbers refer to the peaks in Fig. 1.

FIG. 3. PBP patterns of parental strain COL (lane 1) and glycopeptide-resistant mutants VM (lane 2), TM (lane 3), and TNM (lane 4). The purified plasma membranes were incubated with [3H]benzylpenicillin and were subjected to SDS-polyacrylamide gel electrophoresis and fluorography, as described under “Experimental Procedures.”

RESULTS

Evidence for Decreased Peptide Cross-linking and Increased Glycan Chain Lengths in the Peptidoglycan of Glycopeptide-resistant Mutants.—Fig. 1 shows the HPLC elution profiles of muropeptide species generated from the peptidoglycans of the parental strain COL and its vancomycin-resistant (VM) and teicoplanin-resistant (TM) derivatives, and a third mutant, TM, selected from mutant VM as a highly teicoplanin-resistant derivative (6). The HPLC profiles in panels A show muropeptide species obtained after treatment of the peptidoglycans with the M1 muramidase, an enzyme that breaks glycosidic bonds between the disaccharide units in the peptidoglycan (14). Drastic reduction in the proportion of highly cross-linked muropeptide oligomers (i.e. muropeptide species eluting from the HPLC column with the retention time of muropeptide 17 and with retention times longer) is apparent in each one of the mutant cell walls (see Fig. 1).

Fig. 2 documents in quantitative terms the altered cell wall muropeptide composition in the glycopeptide-resistant mutants. Panels B in Fig. 1 show HPLC profiles obtained after hydrolysis of parental and mutant peptidoglycan with lysostaphin, an enzyme that hydrolyzes the oligoglycine cross-bridges in the peptidoglycan (15, 16). A large increase in the proportion of muropeptide species eluting with long retention times from the reverse phase column is apparent in each one of the resistant mutants.

If one assumed that the lysostaphin digestion resulted in a quantitative breakage of all oligoglycine cross-bridges in the peptidoglycan, then the muropeptide species with the long retention time most likely represented muropeptide monomers attached to glycan chains of increased length as compared with glycan chains in the parental peptidoglycan. This interpreta-
tion was confirmed by the results of double digestion shown in panels C of Fig. 1. It may be seen that a subsequent treatment of the lysostaphin hydrolysates with the M1 muramidase generated a virtually identical set of muropeptides from both parental as well as from the mutant peptidoglycans, and these were identified on the basis of their elution patterns as a group of muropeptide monomers expected to be produced if the lysostaphin digestion preceding the treatment with M1 was complete (11). These observations suggest that the radically decreased peptide cross-linking of the peptidoglycan of the glycopeptide-resistant mutants was accompanied by increase in the average length of the glycan strands.

Expression of PBP4 in the Resistant Mutant—Plasma membrane preparations isolated from the glycopeptide-resistant mutants were tested with the fluorographic assay using [3H]penicillin for the presence of staphylococcal penicillin-binding protein. No PBP4 could be detected in any one of the three highly resistant bacterial mutants (Fig. 3).

Alterations in the DNA Sequence of the PBP4 Determinant of Glycopeptide-resistant Mutants—The \( pbp4 \) gene from the parental strain COL and from mutants VM, TM, and TNM was amplified and sequenced. The sequence of the \( pbp4 \) gene revealed that mutants VM and TM both carried a 17-amino acid duplication at position 305 of the parental gene. In the third

FIG. 4. Amino acid sequence alignment of an internal fragment of PBP4 (amino acids 201–400) of the parental strain COL and glycopeptide-resistant derivatives VM, TM, and TNM. The KTG conserved motif of transpeptidase domain is shown in a box. In the TNM mutant, a point mutation converted the codon coding for Gly-261 into a stop codon while in VM and TM mutants there was a duplication of 17 amino acids (underlined).

FIG. 5. Cell wall hydrolysis in vitro. Cell walls prepared from penicillin-sensitive control strain RN450 from parental strain COL and its glycopeptide-resistant mutants VM, TM, and TNM were degraded by lysostaphin (panel A) and muramidase (panel B). Lysis was measured as a decrease in \( A_{620} \) (OD) during incubation of wall samples.

FIG. 6. Model for the capture of vancomycin molecules in the cell wall of glycopeptide-resistant staphylococci. Glycopeptides are assumed to initiate their antibacterial activity by attachment to the \( \beta \)-alanine-\( \beta \)-alanine termini of cell wall precursors emerging at biosynthetic sites on the bacterial plasma membrane (23–25). Diffusion of drug molecules to the plasma membrane is presumed to occur nearly unhindered through the cell wall of susceptible staphylococci. In contrast, the cell walls of resistant mutants enriched for the muropeptide monomers may slow down and prevent access to the cell wall synthetic sites by attachment of the antibiotic molecules to the \( \beta \)-alanine-\( \beta \)-alanine termini of peptidoglycan monomers. The captured antibiotic molecules may then become part of the mechanism of resistance through the steric hindrance they pose to the penetration of free glycopeptide molecules. CW, cell wall; CM, ; PG, peptidoglycan.
mutant TNM, the alteration in the \( \text{pbp4} \) gene involved introduction of a stop codon into the KTG motif at position 261 in the sequence (Fig. 4).

**Altered Susceptibility of Mutant Cell Walls to Lysostaphin and Muramidase Degradation in Vitro**—The decreased peptide cross-linkage and the apparent increase in the average glycan chain length in the peptidoglycan of the resistant mutants suggested that these alterations may have also caused an alteration in the relative susceptibilities of the mutant cell walls to lysostaphin and the M1 muramidase. This was in fact confirmed. Susceptibility of mutant cell walls to degradation by the M1 muramidase increased, whereas susceptibility to lysostaphin decreased, as compared with the properties of the parental cell wall (Fig. 5). Such a shift in sensitivity would be consistent with the documented decrease in the peptide cross-linking and with the proposed increase in the average glycan chain length in the mutants, because the structural integrity of the mutant cell walls would depend less on the peptide cross-linking network than on the glycan chains.

**DISCUSSION**

The observations described in this study confirm and extend our findings reported earlier (6, 7). The drastically reduced level of peptidoglycan cross-linking, both in the vancomycin-resistant and also in the independently selected teicoplanin-resistant mutants, strongly suggests that this change in peptidoglycan composition is related, directly or indirectly, to the mechanism of antibiotic resistance. Highly cross-linked muropeptides, representing nearly 60% of all muropeptide species in the parental strain, were reduced to about 30, 15, and 17% in mutants VM, TM, and TNM, respectively. Results described in this study strongly suggest that these cell wall alterations are caused by the disruption of \( \text{pbp4} \) in the mutants, resulting in the inactivation or greatly reduced production of PBP4, as evidenced by the negative results of the fluorographic assay.

PBP4 has been shown to have both transpeptidase and D,D-carboxypeptidase activities (17), and this protein was postulated to act in vivo as a secondary transpeptidase required for the extensive cross-linking of peptidoglycan (18). A mutant of \( S. aureus \) lacking \( \text{pbp4} \) (19) was reported to have a hypo-cross-linked peptidoglycan layer (18), as well as a slight increase in susceptibility to \( \beta \)-lactam antibiotics. Other studies (20, 21) demonstrated that overproduction or modification of PBP4 leads to increase in peptidoglycan cross-linking and to increased resistance to methicillin.

Results described in this study indicate that a defect in PBP4 is also associated with the extensive reduction of peptidoglycan cross-linkage in glycopeptide-resistant \( S. aureus \). That this abnormality may be related to the mechanism of resistance is suggested by disruption of \( \text{pbp4} \) by two distinct modes of inactivation: the insertion of a 51-nucleotide sequence near the active site in the mutants with the primary selection for vancomycin resistance; and the introduction of a stop codon at the KTG motif in the mutant selected for by teicoplanin.

The drastic reduction in peptidoglycan cross-linking in the resistant mutants was accompanied by an increased representation of monomeric muropeptides carrying intact carboxy-terminal d-alanyl-d-alanine residues (Fig. 2), which are known to be the recognition sites for glycopeptide antibiotics (22). It was also demonstrated earlier that during the growth of cultures of the glycopeptide-resistant staphylococcal mutants, the bacteria can remove teicoplanin and vancomycin from the medium, and subsequently the sequestered antibiotic can be recovered in biologically active form from the cell walls (6, 7). In the resistant staphylococci, the antibiotic molecules captured by the monomer-rich peptidoglycan may sterically block the porous channels in the cell wall through which incoming drug molecules normally reach sites of cell wall biosynthesis at the plasma membrane (Fig. 6). In this model the diffusion barrier by the captured antibiotic molecules is assumed to become part of the mechanism of resistance.

Although the correlation between the structural abnormality of peptidoglycan and glycopeptide resistance is striking, genetic and biochemical experiments clearly indicate that this cell wall abnormality alone cannot be fully responsible for the mechanism of resistance (6). For instance, whereas cell walls purified from glycopeptide-resistant mutants have clearly increased (2–4-fold) drug binding capacities over that of the cell walls of the parental strain, this increase in binding capacity is much less than what would be expected for the disproportionately large increase in glycopeptide minimal inhibitory concentration value (6, 7). Alterations in the secondary structure of the cell walls and/or changes in other cell surface polymers may also accompany acquisition of glycopeptide resistance (6, 7). Glycopeptide-resistant laboratory mutants carry multistep mutations and show extensive and diverse abnormalities in cell wall metabolism, and the relationship of these to the mechanism of antibiotic resistance remains to be elucidated. One of these unexplained anomalies is the decrease in the methicillin resistance level of mutant VM (6). The mechanism of this observation is not known. However, a similar inverse relationship between the methicillin and vancomycin resistance level was also detected in a set of clinical isolates of methicillin-resistant \( S. aureus \) strain with reduced vancomycin susceptibilities (26).

Another abnormality of wall metabolism was detected by in vitro testing of the susceptibility of mutant cell walls to degradation by lysostaphin and by the M1 muramidase, as described in this study. The tests showed that there was a reversal in the relative sensitivities of the cell walls to these enzymes as compared with the susceptibility of the parental cell walls. Cell walls from the mutants had an increased sensitivity to the M1 muramidase and a decreased sensitivity to lysostaphin, suggesting that the structural integrity of the mutant cell walls has become more dependent on the glycan strands as compared with the peptide cross-linking. The activity responsible for such a structural change is unknown. Nevertheless, this observation is consistent with the results of the experiments illustrated in panels B and C of Fig. 1, which we interpret as an apparent increase in the average glycan chain length in the peptidoglycan of the resistant mutants, perhaps “compensating” for the decrease in peptide cross-linkage.

**REFERENCES**

1. Leclercq, R., Derlot, E., Duval, J., and Courvalin, P. (1988) *N. Engl. J. Med.* 319, 157–161
2. Kaatz, G. W., Seo, S. M. Dorman, N. J., and Lerner, S. A. (1990) *J. Infect. Dis.* 162, 103–108
3. Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T., and Tenover, F. C. (1997) *J. Antimicrob. Chemother.* 40, 135–136
4. Centers for Disease Control (1997) *Morbid. Mortal. Wkly. Rep.* 46, 765–766
5. Centers for Disease Control (1997) *Morbid. Mortal. Wkly. Rep.* 46, 813–815
6. Sieradzki, K., and Tomasz, A. (1997) *Bacteriol. Rev.* 61, 2557–2556
7. Sieradzki, K., and Tomasz, A. (1998) *Microb. Drug Resist.* 4, 159–168
8. Pliska, R. A., and Tomasz, A. (1998) *Bacteriol. J.* 171, 874–879
9. Novick, R. (1967) *Virology* 33, 155–166
10. Sieradzki, K., Villar, P., and Tomasz, A. (1998) *Antimicrob. Agents Chemother.* 42, 100–107
11. de Jonge, B. L. M., Chang, Y.-S., Gage, D., and Tomasz, A. (1992) *J. Biol. Chem.* 267, 11248–11254
12. Laemmli, U. K. (1970) *Nature* 227, 680–685
13. Laskey, R. A., and Mills, A. D. (1975) *Eur. J. Biochem.* 56, 335–341
14. Lichtenstein, A. S. H., Hastings, A. E., Langley, K. E., Mendias, E. A., Rohde, M. F., Elmore, R., and Zukowski, M. M. (1990) *Gene* 88, 81–86
15. Browder, H. P., Zygmunt, W. A., Young, J. R., and Tavormina, P. A. (1965) *Biochem. Biophys. Res. Commun.* 19, 5–9
16. Xu, N., Huang, Z.-H., de Jonge, B. L. M., and Gage, D. A. (1997) *Anaerol. Biochem.* 248, 1–14
17. Kosarich, J. W., and Strominger, J. L. (1976) *J. Biol. Chem.* 253, 1272–1278
Cell Walls in Vancomycin-resistant Staphylococcus aureus

18. Wyke, A. W., Ward, J. B., Hayes, M. V., and Curtis, N. A. C. (1981) Eur. J. Biochem. 119, 389–393
19. Curtis, N. A. C., Hayes, M. V., Wyke, A. W., and Ward, J. B. (1980) FEMS Microbiol. Lett. 9, 263–266
20. Henze, U. U., and Berger-Bachi, B. (1996) Antimicrob. Agents Chemother. 40, 2121–2125
21. Domanski, T. L., de Jonge, B. L. M., and Bayles, K. W. (1997) J. Bacteriol. 179, 2651–2657
22. Nieto, M., and Perkins, H. R. (1971) Biochem. J. 123, 789–803
23. Reynolds, P. E. (1989) Eur. J. Clin. Microbiol. Infect. Dis. 8, 943–950
24. Groves, P., Searle, M. S., Mackay, J. P., and Williams, D. H. (1994) Structure 2, 747–754
25. Mackay, J. P., Gerhard, U., Beauregard, D. A., Westwell, M. S., Searle, M. S., and Williams, D. H. (1994) J. Am. Chem. Soc. 116, 4581–4590
26. Sieradzki, K., Roberts, R. B., Haber, S. W., and Tomasz, A. (1998) N. Engl. J. Med. 340, 517–523