Effect of Exogenous Glycine on Peptidoglycan Composition and Resistance in a Methicillin-Resistant *Staphylococcus aureus* Strain

BOUDEWIJN L. M. DE JONGE,^{1*} Y.-S. CHANG,² N. XU,³ AND D. GAGE³

The Rockefeller University, New York, New York 10021¹; Department of Chemistry, Hanyang University, Ansan, Korea²; and Department of Biochemistry, Michigan State University-National Institutes of Health Mass Spectrometry Facility, East Lansing, Michigan 48824³

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A highly homogeneously methicillin-resistant *Staphylococcus aureus* strain was grown in the presence of various concentrations of exogenous glycine. Increasing concentrations of glycine in the medium resulted in a decrease in methicillin resistance and the appearance of a heterogeneous resistance phenotype. Parallel to the gradual changes in resistance was an alteration in the mucopeptide composition of peptidoglycan. Increasing concentrations of glycine in the medium resulted in peptidoglycan in which mucopeptides with a D-alanyl-D-alanine terminus were replaced with D-alanyl-glycine-terminating mucopeptides. The disappearance of D-alanyl-D-alanine-terminating mucopeptides in peptidoglycan and the concomitant decrease in resistance indicate a central role for D-alanyl-D-alanine-terminating precursors in methicillin resistance.

Resistance to high concentrations of methicillin in *Staphylococcus aureus* is a complex phenomenon that is influenced by multiple genes and growth conditions (1, 10, 15–17). Recently, progress has been made in identifying and understanding the functions of various genes involved in high-level resistance. One gene (*mecA*), which is of nonstaphylococcal origin, encodes a low-affinity penicillin-binding protein (PBP), PBP2A, and is thought to function as a surrogate enzyme when the cell's normal set of peptidoglycan-synthesizing enzymes are inhibited in the presence of β -lactam antibiotics (7, 12). Further studies indicated that additional genes required for high-level resistance are involved in peptidoglycan precursor synthesis. Of the genes known to affect methicillin resistance, two are needed for synthesis of the glycine interpeptide bridge on the lysine residue of the lipid-linked peptidoglycan precursor (5, 6) and a third plays a role in amidation of the glutamate residue of the precursor stem peptide (19). Two additional genes are proposed to be involved in regulation of cytoplasmic peptidoglycan precursor synthesis (5, 20). Hence, biosynthesis of a defined set of peptidoglycan precursors seems to be essential for high-level methicillin resistance in *S. aureus*.

Since high concentrations of exogenous glycine are known to affect peptidoglycan precursor synthesis in *S. aureus* (13, 22, 24), it was reasoned that these growth conditions may also influence methicillin resistance. This hypothesis was tested by growing a highly homogeneous resistant strain in the presence of various concentrations of glycine and determining the effects on methicillin resistance. Peptidoglycan composition was analyzed by high-resolution techniques.

MATERIALS AND METHODS

Strain and growth conditions. The highly homogeneously methicillin-resistant strain *S. aureus* COL (17) was grown in tryptic soy broth (TSB; Difco, Detroit, Mich.) at 37°C at 200 rpm with various concentrations of glycine (Eastman Kodak, Rochester, N.Y.).

Resistance phenotype. Phenotypic expression of resistance was determined by population analysis profiles on tryptic soy agar (TSA; Difco) plates containing various concentrations of methicillin and glycine (9).

Peptidoglycan analysis. Mucopeptide composition of peptidoglycan was ana-

lyzed by reversed-phase high-performance liquid chromatography (HPLC) as described in reference 4.

Mucopeptide analysis. Structures of mucopeptides were determined by a combination of amino acid analysis and fast atom bombardment mass spectrometry (MS) as described previously (4). Tandem MS (MS-MS) experiments were conducted with a JEOL HX 110/110 four-sector instrument. Product ion scans were carried out by using MS1 to select the precursor ($[M+H]^+$ of the mucopeptide) at a resolution of 1,000. Following collision-induced dissociation in a cell located in the field-free region between MS1 and MS2 (collision gas, He; adjusted to reduce the precursor abundance by 50%), the product ion spectrum was obtained by scanning MS2 at a constant B/E ratio. Matrix-assisted laser desorption/ionization MS (MALDI-MS) was carried out on a PerSeptive Biosystems Voyager Elite instrument equipped with a nitrogen laser emitting at 337 nm.

RESULTS

Resistance phenotype. Figure 1A shows population analysis profiles of COL grown on TSA in the presence of various concentrations of glycine. Increased concentrations of glycine in growth medium resulted in heterogeneous expression of resistance with a decrease in methicillin resistance for the majority of cells. Addition of 0.26 M glycine decreased the MIC of methicillin to 3 μ g/ml for most cells (compared with 1,600 μ g/ml in TSA), whereas a minority of the cell population (0.001%) was still able to grow up to a methicillin concentration of 400 μ g/ml.

Two colonies picked from a methicillin-free plate containing 0.26 M glycine were grown to stationary phase in TSB and replated on TSA with various concentrations of methicillin and glycine. As expected, population analysis profiles were the same as those shown in Fig. 1A (data not shown). On the other hand, colonies picked from a plate containing 0.26 M glycine and 12 μ g of methicillin per ml showed different population analysis profiles after growth in TSB and replating on TSA (Fig. 1B). When plated in the presence of 0.26 M glycine, these cells were able to grow on 100 μ g of methicillin per ml and expression of resistance was homogeneous. Continued cultivation of these cells (20 consecutive passages) in drug-free TSB did not change this profile (results not shown).

Addition of equimolar amounts of L-alanine completely prevented the effect of glycine on resistance (Fig. 1C). The addition of the same amount of D-alanine also restored resistance, though to a lesser extent than L-alanine did (Fig. 1C).

Mucopeptide composition of peptidoglycan. To determine the effect of high concentrations of exogenous glycine on

* Corresponding author. Mailing address: Laboratory of Microbiology, The Rockefeller University, Box 152, New York, NY 10021. Phone: (212) 327-8278. Fax: (212) 327-8688.

FIG. 1. Population analysis profiles of strain COL. Cells were grown in TSB to stationary phase and plated at several dilutions on TSA containing various concentrations of methicillin and glycine. After 48 h, the colonies were counted and numbers were expressed as the percentage of cells able to grow on a drug-free plate. % CFU, colony-forming units. (A) COL grown in TSB with 0.00 (■), 0.06 (◆), 0.12 (●), and 0.26 (▲) M added glycine. (B) Highly resistant subpopulation of COL (colony picked from a plate with 12 μ g of methicillin per ml and 0.26 M glycine) grown in TSB with 0.00 (■), 0.12 (◆), and 0.26 (●) M added glycine. (C) COL grown in TSB with 0.00 (■) and 0.26 (▲) M added glycine, with addition of 0.26 M glycine and 0.26 M L-alanine (◆), and with addition of 0.26 M glycine and 0.26 M D-alanine (●).

peptidoglycan, its composition was studied by high-resolution analytical techniques (4). Growth in the presence of glycine resulted in alterations in the mucopeptide composition of peptidoglycan (Fig. 2). Peaks A to F, only present in minute amounts in peptidoglycan of cells grown in TSB, became major components of the peptidoglycan when COL was cultured with added glycine in the growth medium. The relative abundance of these compounds increased with increasing concentrations of glycine (Table 1). With the addition of 0.26 M glycine, peptidoglycan consisted almost exclusively of these compounds. Parallel with the emergence of these "novel" compounds at increasing concentrations of glycine was the disappearance of the hump at the end of the chromatogram (Fig. 2),

indicating the relatively low representation of oligomeric mucopeptides.

The mucopeptide composition of the highly resistant subpopulation of COL (i.e., cells able to grow in the presence of 0.26 M glycine and methicillin concentrations above 12 μ g/ml) did not differ from that of the majority of the cell population (i.e., cells able to grow up to only 3 μ g of methicillin per ml in the presence of 0.26 M glycine) (Table 1).

Characterization of mucopeptides. Structures of the mucopeptides A, B, and C were determined by MS and amino acid analyses (Table 2). The monomeric mucopeptide A contained only two alanine residues (normalized to lysine), in contrast to the three that were reported before for all monomeric mu-

TABLE 1. Mucopeptide compositions of peptidoglycan of *S. aureus* COL grown under a variety of environmental conditions

Peak ^a	Area % (SD) of mucopeptides ^b					0.06 M ^c + 50 μ g of methicillin per ml
	0.00 M ^c	0.06 M ^c	0.12 M ^c	0.26 M ^c	Subpopulation, ^d 0.26 M ^c	
A	0.3 (0.1)	4.6 (0.6)	3.9 (3.3)	3.8 (3.2)	7.8 (0.4)	2.3 (1.2)
1	6.8 (1.1)	0.9 (0.6)	1.5 (0.3)	2.4 (0.3)	0.2 (0.1)	0.7 (0.2)
B	1.8 (0.3)	10.2 (1.7)	17.8 (0.3)	28.2 (0.6)	25.0 (1.9)	9.5 (0.3)
5	11.0 (2.2)	5.9 (0.7)	5.1 (0.7)	4.6 (2.8)	3.1 (0.3)	36.1 (8.6)
C	2.7 (0.4)	11.3 (1.4)	16.2 (0.6)	22.2 (2.0)	20.2 (1.1)	4.9 (1.9)
11	14.8 (0.7)	8.7 (2.0)	5.3 (0.1)	3.4 (1.1)	3.7 (0.2)	24.2 (0.2)
D	3.9 (1.3)	13.6 (0.1)	15.3 (0.3)	15.0 (0.9)	15.7 (0.7)	2.6 (0.3)
15	17.6 (0.6)	9.2 (0.7)	6.4 (1.0)	4.8 (2.6)	3.4 (0.2)	9.2 (2.2)
E	5.2 (0.9)	8.1 (2.8)	9.8 (0.8)	6.2 (3.1)	9.4 (0.5)	1.4 (0.7)
16	15.4 (0.5)	9.5 (1.3)	4.2 (1.9)	2.6 (1.8)	2.0 (0.1)	4.4 (1.7)
F	6.5 (2.7)	9.2 (0.9)	6.7 (1.2)	4.2 (0.8)	6.3 (0.4)	1.8 (1.2)
17	14.0 (3.1)	8.9 (1.3)	7.7 (0.6)	2.9 (0.8)	3.1 (0.3)	3.0 (1.6)
Total (A–F)	20.5 (5.0)	57.0 (5.0)	69.8 (1.7)	79.4 (4.5)	84.4 (1.1)	22.4 (3.1)

^a See Fig. 2.

^b Values are presented as area percentages of the sum of all peaks presented in the table and are the averages for at least two experiments.

^c Amount of glycine added to TSB.

^d Cells able to grow on TSA plate with 12 μ g of methicillin per ml and 0.26 M glycine.

FIG. 2. Separation of *S. aureus* cell wall mucopeptides by reversed-phase HPLC. Peptidoglycan was obtained from strain COL grown in TSB with 0.00 (A), 0.06 (B), 0.12 (C), and 0.26 (D) M added glycine. Mucopeptides were prepared and analyzed after reduction, as described in reference 4. For chemical structures assigned to mucopeptides, see Fig. 4.

ropeptides of *S. aureus* (4). Instead, the amino acid composition of this mucopeptide revealed the presence of one glycine residue, suggesting replacement of one alanine residue in the stem peptide with glycine.

The position at which alanine was replaced with glycine was determined by MS-MS analysis (Fig. 3). The product ion spectrum of the $[M+H]^+$ ion at m/z 954 contained a peak at m/z 909, formed by loss of the terminal carboxyl group. A key a-type fragment (2) at m/z 852 indicated that the C-terminal residue was glycine, and the a-ion at m/z 781 confirmed that the next adjacent residue was alanine. There was no evidence that this order was reversed, since a prominent peak at m/z 838

TABLE 2. Amino acid compositions and molecular masses of mucopeptides isolated from *S. aureus* COL grown in the presence of exogenous glycine

Peak ^a	Amino acid composition ^b				Mol mass ^c (Da)
	Lys	Glx	Ala	Gly	
A	1	0.7	1.9	1.1	954.4
B	1	0.8	2.4	7.3	1,239.5
C	1	1.0	2.7	7.5	2,425.1 ^d

^a See Fig. 2.

^b Molar ratio normalized to Lys. Lys, lysine; Glx, glutamine or glutamate; Ala, alanine; Gly, glycine. All samples contained less than 0.3 mol of serine.

^c $[M+H]^+$ ion of reduced mucopeptide.

^d Upon desalting, this peak was dissolved in several fractions. The molecular mass (Na^+ ion) of the most abundant fraction was obtained by MALDI-MS.

(predicted a-type ion for a terminal alanine) was not present. Other ions in the a-ion series were not apparent in the spectrum, but the remainder of the sequence was determined by fragments representing other ion series. For example, the y-ion at m/z 275 showed that the next residue was lysine, and two z-type ions at m/z 387 and 458 confirmed that isoglutamine and alanine, respectively, were the next two residues in the sequence (Fig. 3).

Mucopeptide B is the pentaglycine-substituted derivative of mucopeptide A (Fig. 4A). MS-MS analysis of this structure also confirmed the presence of a glycine residue at position 5 of the stem peptide (results not shown). The elution time of this mucopeptide was close to or even the same as that of mucopeptide 3. The latter mucopeptide was earlier proposed to be an endopeptidase degradation product, on the basis of the elution time of lysostaphin degradation products and chemical analyses (4). However, since the two compounds have the same chemical composition, proposed structures of compounds eluting at that time need to be verified by MS-MS analysis.

The structure of peak C—the dimeric derivative of compound B—was based on its amino acid compositions and molecular mass and the confirmed structures of the monomeric mucopeptides (Fig. 4A). On the basis of retention times, peaks D, E, and F are consistent with tri-, tetra-, and pentameric compounds of structure B.

Peptidoglycan composition of cells grown in the presence of methicillin and glycine. To test for PBP2A's substrate specificity, COL was grown in TSB supplemented with 0.06 M glycine and 50 μ g of methicillin per ml. These conditions were chosen because at this concentration of glycine, D-alanyl-D-alanine- as well as D-alanyl-glycine-terminating precursors are synthesized (Fig. 2B) and the concentration of methicillin saturates all PBPs except PBP2A (3). Peptidoglycan synthesized under these conditions can be considered the product of PBP2A.

Under these conditions, a striking change in the mucopeptide profile was observed in comparison with the profile obtained by growth in the presence of 0.06 M glycine only (Fig. 5). The highly oligomeric species (the hump at the end of the chromatogram) disappeared, and mucopeptides with a glycine residue in the stem peptide (peaks A to F) were present only in small amounts (Table 1). The major mucopeptides present in peptidoglycan synthesized under these conditions were pentaglycine-substituted mono- and dimers containing three alanine residues in the stem peptide (peaks 5 and 11; Fig. 4B). This mucopeptide profile was almost identical to that of cells grown in TSB in the presence of 50 μ g of methicillin per ml without addition of glycine (7).

FIG. 3. MS-MS spectrum of peak A. The product ion scan of the $[M+H]^+$ ion at m/z 954 displays diagnostic fragments for the assignment of the proposed structure.

DISCUSSION

Peptidoglycan composition. It has been shown that in *S. aureus* grown with high concentrations of glycine in the medium, this amino acid can compete with alanine for positions 1, 4, and 5 of the peptidoglycan precursor stem peptide at the cytoplasmic stage of peptidoglycan precursor synthesis, but as a consequence of a discrimination in the latter steps of peptidoglycan biosynthesis, mainly species with a glycine residue at position 5 are found in (mature) peptidoglycan (13, 18, 22, 24). Using high-resolution techniques to obtain more-detailed information about the peptidoglycan composition, this study also confirmed that in methicillin-resistant *S. aureus*, mainly mucopeptides with a carboxyl-terminal glycine residue are found in peptidoglycan when cells are grown in the presence of high concentrations of glycine.

Throughout the concentration range of exogenous glycine tested, only mucopeptides with glycine residues in position 5 were detected in peptidoglycan, but the relative abundance depended on the concentration of glycine. Peptidoglycan of cells grown in the presence of 0.06 M glycine contained about equal amounts of mucopeptides with a D-alanyl-D-alanine terminus and with a D-alanyl-glycine terminus, whereas the mucopeptide composition of peptidoglycan synthesized at higher concentrations of glycine contained almost no D-alanyl-D-alanine-terminating species. This could be reversed by addition of alanine to the medium. The increased amounts of glycine-terminating mucopeptides in peptidoglycan with increasing concentrations of glycine in the medium are likely a reflection of the ongoing competition that occurs at the level of cytoplasmic precursor synthesis.

Methicillin resistance. Addition of exogenous glycine to growth medium also led to a profound reduction in methicillin resistance and caused (at concentrations above 0.06 M) a heterogeneous phenotype. Heterogeneous phenotypes are common among clinical isolates and have been reported to occur in transposon mutants of highly resistant strains (1, 11, 14, 15, 25), but the phenomenon is still poorly understood (16). It remains unclear why mutations that resulted in abnormalities in peptidoglycan composition (*femA*, *femB*, *femC*, and *femF* mutants) are associated with heteroresistance (5, 6, 19, 20). Also, these mutations are unlikely to play a role in heteroresistance observed to occur in clinical isolates. It has been proposed that other chromosomal mutations—located outside the *mec*-associated DNA region and the *femAB* operon—may be involved (21). Thus, elucidating the functions of the many (still unstudied) genes that are known to affect methicillin resistance (10) may provide more insights into this complex phenomenon. Besides the genotype, environmental factors have also been implicated in heterogeneous expression of resistance (16). This study identified high concentrations of glycine as one of the factors, but the mechanism by which addition of glycine resulted in such a phenotype is still unclear.

This study confirmed the finding that highly resistant subpopulations become homogeneously resistant when (re)grown and (re)plated (9, 23) (Fig. 1B). Because the resistance profile of the subpopulation was stable for at least 200 generations, alterations in the genotype leading to highly resistant subpopulations must have occurred. Mutations giving rise to highly resistant subpopulations have been reported before (21). The nature of the mutation(s) remains to be determined, but (in

FIG. 4. Chemical structures of mucopeptides predominantly present in peptidoglycan synthesized in medium containing an excess of glycine (A) or in TSB without addition of glycine (B). Structures are based on amino acid composition, molecular mass (Table 2), and MS-MS analysis (peaks A and B). Structures of peaks 1, 5, and 11 are taken from reference 4. G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid, ALA, alanine; GLX, glutamine; LYS, lysine; GLY, glycine.

this study) the mutation did not affect peptidoglycan composition. A possible mutation in one of the genes encoding PBPs could be compatible with an altered level of resistance and changed peptidoglycan precursor synthesis, a situation analogous to that observed with penicillin-resistant pneumococci (12a). It remains to be determined if such a mutation occurred, and if so whether it occurred in the genes encoding one of the four staphylococcal PBPs or in the *mecA* gene encoding PBP2A.

The decrease in resistance for the majority of COL cells grown in the presence of high concentrations of glycine was accompanied by alterations in peptidoglycan composition, and we hypothesize that the two phenomena are linked. Correlation of alteration in peptidoglycan composition and reduction in methicillin resistance was reported before, for a set of Tn551 mutants of a highly resistant strain (5, 6, 19, 20). Thus, genotypic (Tn551 mutants) as well as phenotypic (this study) alterations showed an association between peptidoglycan composition and methicillin resistance. As with the Tn551 mutants, alterations in peptidoglycan composition caused by high concentrations of glycine in the medium are presumably due to changed peptidoglycan precursor synthesis.

It was recently proposed that for high-level resistance to be expressed by COL, an appropriate peptidoglycan precursor has to be provided to PBP2A, the protein that is presumed to allow peptidoglycan synthesis to continue under conditions at which all other PBPs become saturated with antibiotics. On the basis of the peptidoglycan composition of Tn551 mutants in COL, the preferred precursor contained a pentaglycine-substituted pentapeptide with a glutamine residue at position 2 (8). The decrease in methicillin resistance when the organism is synthesizing D-alanyl-glycine- instead of D-alanyl-D-alanine-terminating pentapeptide precursors, i.e., at high concentrations of glycine in the medium, can be interpreted as an inability of PBP2A to use the glycine-terminating precursor as a substrate. This would thus limit PBP2A's substrate specificity to a pentaglycine-substituted pentapeptide with a D-alanyl-D-alanine carboxy terminus and a glutamine residue at position 2. When the availability of this precursor reaches a critical level (above

0.06 M exogenous glycine in the medium) PBP2A will not be able to function properly and resistance will decrease.

This proposed substrate specificity was confirmed by the analysis of peptidoglycan synthesized in TSB supplemented

FIG. 5. Separation of *S. aureus* cell wall mucopeptides by reversed-phase HPLC. Peptidoglycan was obtained from strain COL grown in TSB with the addition of 0.06 M glycine (A) and in TSB with the addition of 0.06 M glycine and 50 μ g of methicillin per ml (B). Mucopeptides were prepared and analyzed after reduction as described in reference 4. Chemical structures assigned to mucopeptides are shown in Fig. 4.

with 0.06 M glycine and 50 µg of methicillin per ml. Peptidoglycan synthesized under these conditions, which can be considered the product of PBP2A, was composed almost exclusively of D-alanyl-D-alanine-terminating monomers and dimers (peaks 5 and 11) and contained only minute amounts of D-alanyl-glycine monomers (peaks A and B). Assuming that methicillin did not affect the mixed cytoplasmic precursor synthesis, these results show that PBP2A has an almost exclusive preference for D-alanyl-D-alanine-terminating species over D-alanyl-glycine species in the transpeptidation reaction.

Environmental conditions and methicillin resistance. This study identified yet another environmental parameter that affects methicillin resistance: high concentrations of glycine. Since alanine was able to prevent the effect of glycine, the ratio of alanine to glycine in the medium may be a determinant in methicillin resistance. Because of the high concentrations involved, this is unlikely to be a reason for discrepancies in sensitivity testing with different growth media.

Although it may not be the only factor involved, the studies described here support the hypothesis that biosynthesis of a specific set of peptidoglycan precursors is essential for high-level methicillin resistance in *S. aureus*. Further studies are required to determine whether other environmental conditions that influence methicillin resistance act by a similar mechanism(s).

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