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Mechanism of Resistance to Vancomycin in *Enterococcus faecium* D366 and *Enterococcus faecalis* A256

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The role of the glycopeptide-inducible proteins of *Enterococcus faecium* D366 (39.5 kilodaltons) and *Enterococcus faecalis* A256 (39 kilodaltons) in the mechanism of resistance to vancomycin and teicoplanin was examined. Crude cell walls from noninduced cells or from induced cells treated with sodium dodecyl sulfate to remove the inducible proteins were shown to bind vancomycin, in contrast to cell walls containing the cytoplasmic membrane-associated induced proteins, which did not bind vancomycin. Cytoplasmic membranes from vancomycin-induced cells did not inactivate (bind) vancomycin or teicoplanin, but they could protect the glycopeptides from being bound to the synthetic pentapeptide. This protection could be competitively abolished by D-alanyl-D-alanine. A decrease in glycopeptide binding to the pentapeptide was observed in a time-dependent fashion after treatment of the pentapeptide with the cytoplasmic membranes from induced cells. We hypothesize that the inducible proteins are responsible for glycopeptide resistance due to the binding to, and subsequent enzymatic modification of, the pentapeptide precursor of peptidoglycan, which is considered to be the natural target of glycopeptides.

Recently, resistance to vancomycin has been found in clinical isolates of *Enterococcus faecium* and *Enterococcus faecalis* (5, 6, 13, 15, 16). This resistance was shown to be inducible, in most cases transferable (5, 6, 13), and sometimes plasmid mediated (5, 6). A correlation has been observed between resistance and the appearance or increased synthesis of proteins of either 39.5 (16) or 39 kilodaltons (kDa) (8, 13) which are also inducible by a variety of other glycopeptides (12, 13, 16). In this report, we experimentally confirm that the presence of these proteins in large quantities in the cytoplasmic membrane reduces the availability of the peptidoglycan pentapeptide which has been reported to be one of the main targets of the glycopeptides (1, 3, 4, 9, 10, 14).

MATERIALS AND METHODS

Bacterial strains and growth medium. *E. faecium* D366 and *E. faecalis* A256 are two vancomycin-resistant strains previously reported (13, 16). MICs of vancomycin and teicoplanin were, respectively, 64 and 0.5 μg/ml for *E. faecium* D366 and 256 and 16 μg/ml for *E. faecalis* A256. Cultures of these strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.).

Antibiotics and bioassay. Vancomycin was provided by Eli Lilly & Co., Indianapolis, Ind., and teicoplanin was provided by Lepetit Research Center, Milan, Italy. Vancomycin and teicoplanin were quantified by a microbiological assay with *Sarcina lutea* ATCC 9341 as an indicator strain. For each experiment a reference curve was constructed with concentrations ranging from 0.2 to 200 μg/ml. Concentrations of 0.5 μg of each of the antibiotics per ml were reliably detected. All bioassays of glycopeptides were done in a final volume of 30 μl.

Analysis of proteins from cell walls and cytoplasmic membranes. The cells from 200 ml of culture in the logarithmic phase of growth (optical density at 650 nm, ca. 0.25) were harvested by centrifugation at 9,000 × *g* at 4°C for 10 min. After being washed and suspended in sodium phosphate buffer (50 mM, pH 7), the cells were disrupted with glass beads in a cell disintegrator (The Mickle Laboratory Engineering Co., Gomshall, United Kingdom). Cell walls were collected by centrifugation at 4,000 × *g* for 10 min, and cytoplasmic membranes were pelleted from the supernatant at 100,000 × *g* for 30 min and stored at −70°C (13, 16). Wall and membrane fractions were washed three times in buffer. Cell walls or membranes were boiled for 2 min in 2% sodium dodecyl sulfate (SDS), and the proteins were analyzed by electrophoresis in SDS-containing polyacrylamide (10%) gels. Proteins were stained with Coomassie blue.

Induction of the 39.5- and 39-kDa proteins. The 39.5- and 39-kDa proteins were induced after growth of the cells in the presence of vancomycin (8 μg/ml [13, 16]). When cell wall or membrane proteins were to be extracted from induced cells, the overnight cultures were diluted 100-fold and grown, at the same concentration of vancomycin, to the logarithmic phase as described above.

Binding of glycopeptides to the cell wall. Amounts of cell walls were estimated via the determination of cell wall-associated proteins by the method of Lowry et al. (7) and expressed as protein equivalents (PEq). Identical amounts from noninduced or induced cells of strains D366 and A256 were treated in two ways. One set was boiled in 4% SDS (to remove all cell wall-associated proteins), washed, and centrifuged (100,000 × *g*, 30 min) three times in distilled water at 20°C. The other set was simply washed under the same conditions. Cell walls (0 to 3.3 mg [PEq]/ml of phosphate buffer, 50 mM, pH 7), were incubated with 33 μg of vancomycin per ml for 15 min at 20°C. Active (i.e., unbound) vancomycin was directly determined by the bioassay.

Binding of glycopeptide to pentapeptide. Pentapeptide (Ala-D-γ-Glu-Lys-D-Ala-D-Ala, 3 mM; Sigma Chemical Co., St. Louis, Mo.) was incubated (30 min, 20°C) with 0 to 3.3 mg [PEq] of cytoplasmic membrane per ml (determined as for cell walls) from noninduced or induced cells. Thereafter, glycopeptides (33 μg/ml) were added. After an additional 15 min, the glycopeptide which remained unbound to the pen-

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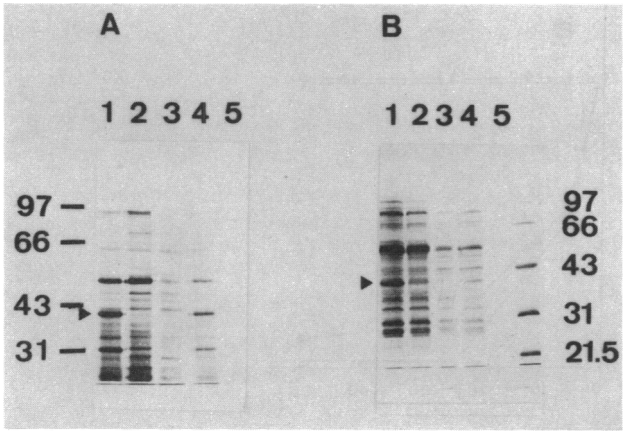

FIG. 1. Protein patterns of CWM (see Results) and cytoplasmic membranes. (A) *E. faecium* D366; (B) *E. faecalis* A256. Lanes: 1, induced cytoplasmic membranes; 2, noninduced cytoplasmic membranes; 3, noninduced CWM; 4, induced CWM; 5, induced and SDS-treated CWM. The positions of the 39.5-kDa (A) and 39-kDa (B) proteins are indicated. The A256 protein comigrating at the 39-kDa position (panel B, lanes 2 and 3) was found to be distinct from the induced protein after two-dimensional polyacrylamide gel electrophoresis (data not shown).

tapeptide was quantified by the bioassay. To assay whether glycopeptide binding to the pentapeptide was time dependent under these conditions, pentapeptide was incubated for 1 min to 4 h with cytoplasmic membranes before the addition of the glycopeptides. All binding experiments were carried out at least twice, and variation was less than 10% for each value obtained.

RESULTS

Binding of vancomycin to cytoplasmic membranes and cell walls. When cytoplasmic membranes (0.03 to 3.3 mg [PEq]/ml, i.e., 1 to 100 μg per assay in 30 μl) from noninduced or induced cells were incubated with vancomycin (33 μg/ml, 12 μM), no more than a 3 to 4% decrease in vancomycin activity was observed (data not shown), indicating that no binding to or inactivation by the cytoplasmic membrane fraction occurred.

Cell walls isolated after cell disruption with glass beads contained some cytoplasmic membranes even after repeated washing, as inferred from the protein analysis shown in Fig. 1. We therefore named them cell wall-membrane fractions (CWM). After induction of cells by vancomycin, the CWM contained large quantities of the inducible proteins (13, 16) of 39.5 kDa in *E. faecium* D366 and 39 kDa in *E. faecalis* A256 (Fig. 1).

When increasing amounts of CWM from noninduced *E. faecium* D366 or *E. faecalis* A256 were incubated with vancomycin (33 μg/ml), the quantities of free vancomycin decreased (Fig. 2). When 30 μg (PEq) of CWM per 30 μl was used, close to 80 or 100% of the vancomycin was bound to CWM from noninduced strain D366 or A256, respectively. By contrast, in the same experiment, with CWM of the two strains after induction, no more than 20% of the vancomycin was bound, even when 100 μg (PEq) of CWM per 30 μl was used.

To assess the effect of the cytoplasmic membrane containing the induced proteins on the binding of vancomycin to the cell wall, samples of CWM previously boiled in 4% SDS for 30 min were used. No proteins were present in the boiled sample (Fig. 1), demonstrating that this procedure had resulted in the removal of the cytoplasmic membrane from the CWM. After incubation of these purified cell walls with vancomycin under the conditions described above, significant binding of vancomycin could be demonstrated for the two organisms which was almost as pronounced as that seen with the CWM of noninduced *E. faecium* D366 (Fig. 2). Similar results were obtained under the same conditions with teicoplanin (data not shown).

Inhibition of vancomycin and teicoplanin binding to the pentapeptide. Previous experiments have shown that different polypeptides with a terminal D-alanyl-D-alanine (D-Ala-D-Ala), including the pentapeptide used in this study, bind vancomycin (9, 11). Incubation of the pentapeptide (0.1 to 10 mM) with vancomycin or teicoplanin (33 μg/ml) for 30 min resulted in total inactivation of the two glycopeptides (as measured by the bioassay) with 3 mM pentapeptide.

We examined the possible protective role of the membrane from induced cells on the binding of vancomycin to the pentapeptide. In the presence of 50 μg (PEq) of membrane from noninduced *E. faecium* D366 or *E. faecalis* A256 per 30 μl, more than 95% of the vancomycin was bound to the pentapeptide at 3 mM (Fig. 3). In contrast, in the presence of

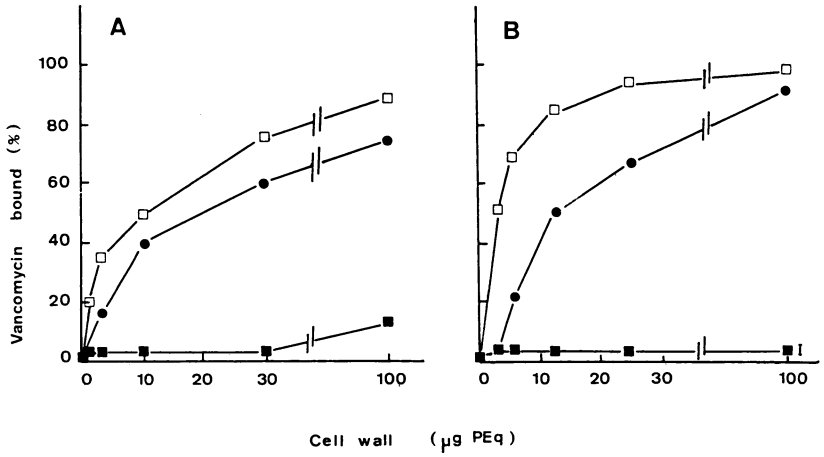

FIG. 2. Vancomycin binding to CWM. (A) *E. faecium* D366; (B) *E. faecalis* A256. Results are shown for noninduced (□), induced (■), and SDS-treated induced (●) CWM. The amounts of CWM used per assay (in 30 μl) are given in micrograms (PEq).

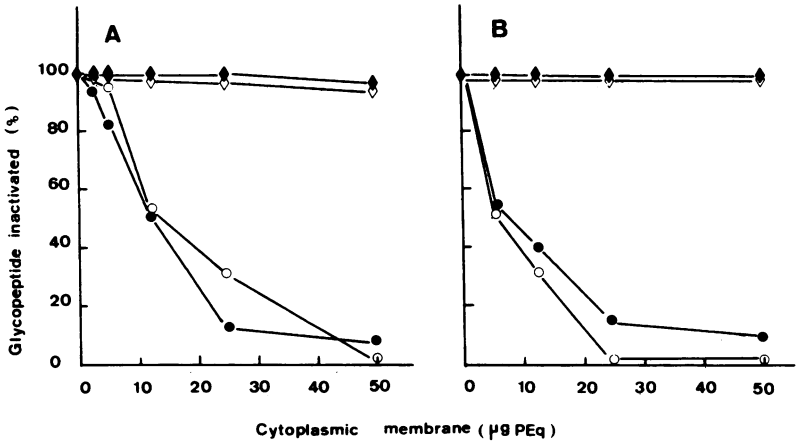

FIG. 3. Inhibition of glycopeptide binding to pentapeptide by cytoplasmic membranes. (A) *E. faecium* D366; (B) *E. faecalis* A256. Noninduced (◆, ◇) and induced (●, ○) membranes were incubated with pentapeptide (3 mM, 10 min, 20°C) and subsequently with vancomycin (◆, ●) or teicoplanin (◇, ○). The amounts of cytoplasmic membranes used per assay (in 30 µl) are given in micrograms (PEq).

membrane from the induced cells, there was a very significant decrease of binding to the pentapeptide, with less than 10% of the vancomycin bound in the presence of 25 µg (PEq) of membrane per 30 µl. Since *E. faecium* D366, in contrast to *E. faecalis* A256, does not show any increase in the MIC of teicoplanin (13, 16), we examined whether membranes from the two induced strains would also affect the binding of teicoplanin to the pentapeptide. The two membranes reduced binding of teicoplanin in a manner similar to that observed with vancomycin (Fig. 3).

Time-dependent inactivation of the pentapeptide capacity for binding glycopeptides. In the previous experiment (Fig. 3), 1.7 mg (PEq) of induced membranes per ml almost completely inactivated 3 mM pentapeptide (M_r, 488). Assuming that the inducible proteins (39.5 or 39 kDa) represented approximately 10 to 20% of the cytoplasmic membrane proteins (Fig. 1), it can be estimated that each induced protein molecule interacted with several hundred molecules of pentapeptide under the experimental conditions. Since such a ratio appears to be incompatible with simple binding, we examined whether the inactivation of the pentapeptide binding capacity was time dependent. In the presence of 10 µg (PEq) of cytoplasmic membranes from the vancomycin-induced strains *E. faecium* D366 and *E. faecalis* A256, there was a time-dependent inactivation of pentapeptide, which did not occur to a great extent with membranes from noninduced cells (Fig. 4). This finding suggests an enzymatic process inactivating the pentapeptide. This assumption was further supported by the observation that induced cytoplasmic membranes from *E. faecium* D366 after heating at 50°C for 15 min and from *E. faecalis* A256 after heating at 100°C for 5 min were unable to prevent binding of vancomycin to the pentapeptide (data not shown).

Role of the D-Ala-D-Ala moiety. To assess whether D-Ala-D-Ala, which is the terminal moiety of the pentapeptide, could bind to the inducible proteins, the following experiment was designed. Cytoplasmic membrane containing the induced protein of 39.5 kDa (1.6 mg [PEq]/ml) was incubated with D-Ala-D-Ala (1 to 32 mM) at 20°C for 10 min. Then pentapeptide (3 mM) was added and incubation was continued for another 10 min. Finally, vancomycin (33 µg/ml) was added. Increasing concentrations of D-Ala-D-Ala were correlated with increasing inactivation of vancomycin (Fig. 5). Identical results were obtained with cytoplasmic membranes containing the 39-kDa proteins (data not shown). Since D-Ala-D-Ala alone did not inactivate (bind) vancomycin, a

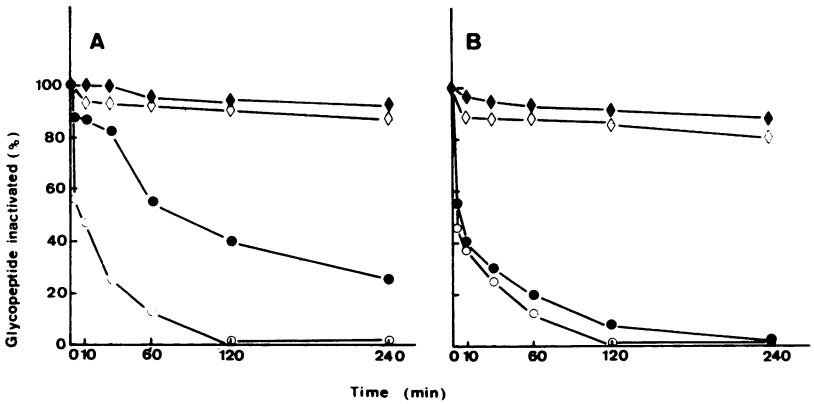

FIG. 4. Time-dependent inactivation of the pentapeptide by cytoplasmic membranes and subsequent inhibition of glycopeptide binding. (A) *E. faecium* D366; (B) *E. faecalis* A256. Noninduced (◆, ◇) and induced (●, ○) membranes were incubated with pentapeptide (3 mM, 1 min to 4 h, 20°C) and subsequently with vancomcyin (◆, ●) or teicoplanin (◇, ○). The amount of cytoplasmic membranes used per assay (in 30 µl) was 10 µg (PEq).

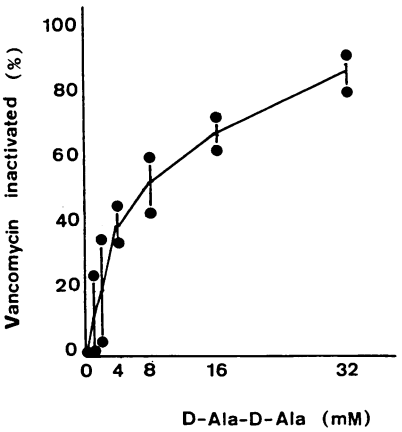

FIG. 5. Binding of D-Ala-D-Ala to the induced cytoplasmic membrane of *E. faecium* D366. The amount of inactivated vancomycin reflects the amount of pentapeptide not bound to the induced cytoplasmic membrane when the latter was preincubated with dipeptide.

likely explanation would be that the induced protein bound the D-Ala-D-Ala instead of the pentapeptide, which would then remain unmodified and be free to inactivate the vancomycin.

DISCUSSION

Glycopeptides have been shown to inhibit the synthesis of peptidogolycan and cause the accumulation of the UDP-*N*-acetyl-muramylpentapeptide (1, 3, 4, 11). The exact mechanism of this inhibition is not clear, but it has been demonstrated that vancomycin binds to cell wall intermediates containing sugar-linked precursor UDP-*N*-acetyl-muramylpentapeptide or its lipid carrier or both (11). Other studies have also clearly shown that binding of vancomycin to cell wall intermediates specifically involves the recognition of the L-Lys-D-Ala-D-Ala terminus of the pentapeptide (3, 9).

In previous studies, it was shown that the emergence of inducible vancomycin resistance in *E. faecium* and *E. faecalis* was correlated with the induction of the synthesis of two cytoplasmic membrane proteins of 39.5 and 39 kDa, respectively, and it was suggested that these proteins could impede the access of vancomycin to its target (12, 13, 16).

In this study, we have taken up the suggestion. We have shown that cell walls with associated membranes from *E. faecium* D366 and *E. faecalis* A256 which had been induced by vancomycin at low concentrations did not bind vancomycin but that cell walls from noninduced cells or from vancomycin-induced cells after being boiled in 4% SDS did bind vancomycin. However, from this experiment it is not possible to distinguish between protection of the target site or protection against nonspecific binding to the cell wall (1).

Using membranes from induced and noninduced cells, we have shown that only membranes from induced cells prevented binding of vancomycin to exogenous pentapeptide and that this process was time dependent and could be inhibited with D-Ala-D-Ala. Similar results were obtained when teicoplanin was used instead of vancomycin.

We consider these results to demonstrate that a component of the cytoplasmic membrane from vancomycin-induced cells of both *E. faecium* D366 and *E. faecalis* D256 does indeed diminish the access of glycopeptides to the pentapeptide in the natural cell walls of these organisms. Since cytoplasmic membrances from noninduced and induced cells have quite similar protein compositions, with the exception of notably larger amounts of the 39- or the 39.5-kDa protein, we infer that these proteins are involved in the absence of binding to the glycopeptide target site(s). Since the cytoplasmic membranes from induced cells did not bind glycopeptides, since the estimation of the stoichiometry of the induced protein-pentapeptide interaction indicated a high molar excess of pentapeptide over protein, since the pentapeptide inactivation by the induced proteins was time dependent, and since this inactivation could be abolished by heat inactivation of the induced proteins, we suggest that the most likely mechanism of glycopeptide resistance is the binding of the induced proteins to the pentapeptide and its subsequent modification in such a way that glycopeptide binding is prevented. This process probably occurs via the terminal D-Ala-D-Ala moiety of the pentapeptide, as suggested by the competition experiment with the dipeptide. *E. faecium* D366 is susceptible to teicoplanin, which does not induce synthesis of the 39.5-kDa protein. However, resistance to this drug is vancomycin inducible (16). Consistent with this finding is the observation that membrane preparations from vancomycin-induced cells do not allow the binding of teicoplanin, just as they do not allow the binding of vancomycin, to the pentapeptide, and apparently to the same degree (Fig. 3). The definitive confirmation of the role of the 39- and 39.5-kDa proteins in glycopeptide resistance will, of course, require the use of the isolated inducible proteins in similar experiments.

The original function of these proteins has yet to be defined. However, it can be speculated that the pentapeptide-binding sites might resemble those of enzymes able to bind D-Ala-D-Ala, e.g., enzymes involved in the metabolism of the dipeptide or the pentapeptide or in the polymerization of the disaccharide-pentapeptide. If one assumes that there is processing of the pentapeptide, as suggested by the time-dependent inactivation of the pentapeptide, and that it occurs at the D-Ala-D-Ala terminus, the function(s) of these proteins resembles those of a carboxypeptidase or of a transpeptidase. Preliminary results (data not shown) have demonstrated that incubation of pentapeptide with membrane proteins from induced cells of *E. faecium* D366 and *E. faecalis* A256 caused a substantially increased release of free alanine (as compared with that observed with proteins from noninduced cells). This argues for an enzymatic process possibly hydrolyzing the terminal D-Ala-D-Ala. As a consequence, there should be no endogenous pentapeptide for the glycopeptides to bind and to allow inhibition of the terminal stage of peptidoglycan synthesis. We have no information yet about the type and the extent of peptidoglycan crosslinkage under these conditions.

With the mechanism of resistance now reasonably apparent, the mechanism of induction remains obscure. Considering a similar function for the two proteins of *E. faecium* and *E. faecalis*, one might expect structural similarities between them. If they exist, they do not seem to be extensive, as judged from preliminary immunochemical analysis (S. Al-Obeid, L. Gutmann, D. Shlaes, and E. Collatz, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 274, 1989). The question remains whether the structural differences have any bearing on the differences observed in the patterns of inducibility.

It has also been shown that after synthesis of these proteins glycopeptide resistance is accompanied by increased susceptibility to penicillins (13, 16). One possibility is that the proteins, after binding to and modifying the

endogenous pentapeptide, do not allow normal synthesis of the peptidoglycan by the cell wall enzymes (i.e., transpeptidases or carboxypeptidases), as might also be inferred from the altered cell shape of vancomycin-induced strains (16). Inhibition only of those penicillin-binding proteins with a high affinity for penicillin and still active in cross-linking might then be sufficient to inhibit total cell wall synthesis (2). Analysis of the penicillin-binding proteins and of the peptidoglycan of a constitutive vancomycin- and teicoplanin-resistant, but penicillin-susceptible, mutant of *E. faecium* D366 producing large amounts of the 39.5-kDa protein (S. Al-Obeid, unpublished observation) should allow us to test our hypothesis.

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