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Penicillin Tolerance and Modification of Lipoteichoic Acid Associated with Expression of Vancomycin Resistance in VanB-Type *Enterococcus faecium* D366

LAURENT GUTMANN,[1]* SULEIMAN AL-OBEID,[1]† DANIÈLE BILLOT-KLEIN,[1]
EDELTRAUD EBNET,[2] AND WERNER FISCHER[2]

Laboratoire de Recherche Moléculaire sur les Antibiotiques, Université Paris VI, 75270 Paris Cedex 06, France,[1] and Institut für Biochemie, Universität Erlangen Nürnberg, D-91054 Erlangen, Germany[2]

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Induction of vancomycin resistance in *Enterococcus faecium* D366, which exhibits a VanB-type resistance, as well as its constitutive expression in MT9, a derivative of D366, was associated with penicillin tolerance as shown by decreased lysis and killing of the cells. This phenomenon was linked neither to decreased expression of the different autolysins nor to their decreased lytic activity on the different cell walls. The only change observed was that almost twice the normal amount of D-alanine was attached to the lipoteichoic acid.

Resistance to glycopeptides in *Enterococcus faecium* of the VanB class is characterized by resistance to vancomycin and susceptibility to teicoplanin, with resistance being inducible by vancomycin but not by teicoplanin (30). The mechanism of VanB resistance is explained by the presence of acquired genes (3, 10, 18) that are responsible for the synthesis of a new peptidoglycan precursor (6) ending in D-lactate (pentadepsipeptide) (7), which has a low affinity for the glycopeptides (3, 25). Lytic and bactericidal effects of penicillin on the VanB-class *E. faecium* D366 before and after induction of the vancomycin resistance and on its spontaneous constitutive mutant MT9 were studied.

Cultures were grown at 37°C in brain heart infusion broth or on brain heart infusion agar (Sanofi Pasteur Diagnostics). Lytic effects and titers of viable cells were determined after the addition of antibiotic to exponential-phase cultures at an optical density at 650 nm of 0.2 (approximately 10^8 bacteria per ml). The spontaneous lysis of intact cells (2) was detected with cells grown at 37°C at an optical density at 650 nm of 0.2, centrifuged at 10,000 × *g* (4°C), washed once, and resuspended at their initial volume in 50 mM phosphate buffer (pH 5.7). Autolytic activity was assayed as previously described (17), with heat-killed *E. faecium* cells as substrate in brain heart infusion agar: ten microliters of an overnight culture tested for hydrolase activity was spotted on the surface of the medium and incubated at 37°C for 48 h. Autolytic enzymes were detected, according to the method of Beliveau et al. (4), on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel to which 1 mg of dry, heat-inactivated *E. faecium* cells per ml had been added. Samples corresponding to 2 ml of *E. faecium* grown to an optical density at 650 nm of 0.4 were resuspended in 30 μl of phosphate buffer (50 mM, pH 7) and lysed with 10 μg of M1 muramidase for 15 min at 37°C. Renaturation of lytic enzymes present in the samples was done by overnight incubation of the gel in 25 mM Tris-HCl (pH 8) containing 1% (vol/vol) Triton X-100 at 37°C.

Lipoteichoic acid (LTA) was extracted and purified under conditions that preserve the native substitution with D-alanine ester (12, 13, 15). Analyses of LTA for chain structure, lipid anchor, fatty acid composition, D-alanine ester, glycosylated glycerol moieties, and chain length were done as previously described (11, 13, 14).

E. faecium D366 (30) is a low-level resistant, vancomycin-inducible VanB-type strain (MIC of vancomycin, 32 μg/ml) that is susceptible to teicoplanin. The MIC of penicillin for this strain was 16 μg/ml but dropped to 0.25 μg/ml once the resistance was induced by the presence of 4 μg of vancomycin per ml. MT9 is a constitutive mutant resistant to glycopeptides (21, 28) derived from D366, with MICs of vancomycin and teicoplanin of 64 and 8 μg/ml, respectively. It was hypersusceptible to penicillin (MIC, 0.25 μg/ml), as has been previously described for other constitutive mutants (1, 20).

When eight times the MIC (128 μg/ml) of penicillin was used, lysis of D366 (Fig. 1) was associated with a 2-log decrease in CFU after 4 h. When D366 was first induced with vancomycin (4 μg/ml) for 3 h and then exposed (still in the presence of vancomycin to maintain the expression of vancomycin resistance) to the same concentration of penicillin (128 μg/ml), only slight lysis and almost no decrease in CFU were observed. With the constitutive, resistant mutant MT9 and penicillin at eight times the MIC (2 μg/ml) or at 128 μg/ml and in the absence of vancomycin, neither lysis nor killing was observed. This suggested that, similarly to what was observed with the induced D366 strain, the expression of glycopeptide resistance was associated with tolerance to penicillin. Interestingly, after resuspension of the induced strain D366 and of mutant strain MT9 in phosphate buffer, spontaneous lysis occurred less rapidly and to a lesser extent than that of noninduced strain D366 (data not shown).

We addressed the question of whether some changes in the autolysins in these strains had occurred. With Mueller-Hinton agar containing heat-killed MT9 cells, noninduced D366 cells, and induced D366 cells as substrate, a similar halo of lysis was obtained with living D366 or MT9 cells (data not shown). When SDS-polyacrylamide gel electrophoresis was performed on gels containing heat-killed D366 cells, three major autolysins, of about 25, 33, and 85 kDa and present in apparently similar quantities, were found in crude extracts of MT9 and noninduced and induced D366 cells (Fig. 2). When heat-killed cells of the induced D366 strain or MT9 were used as substrate, the same autolysin pattern was obtained (data not shown).

* Corresponding author. Mailing address: Université Paris VI, L.R.M.A., 15, rue de l'Ecole de Médecine, 75270 Paris Cedex 06, France. Phone: (33)-1-43.29.28.63.

† Present address: King Fahed Central Hospital, Gizan, Saudi Arabia.

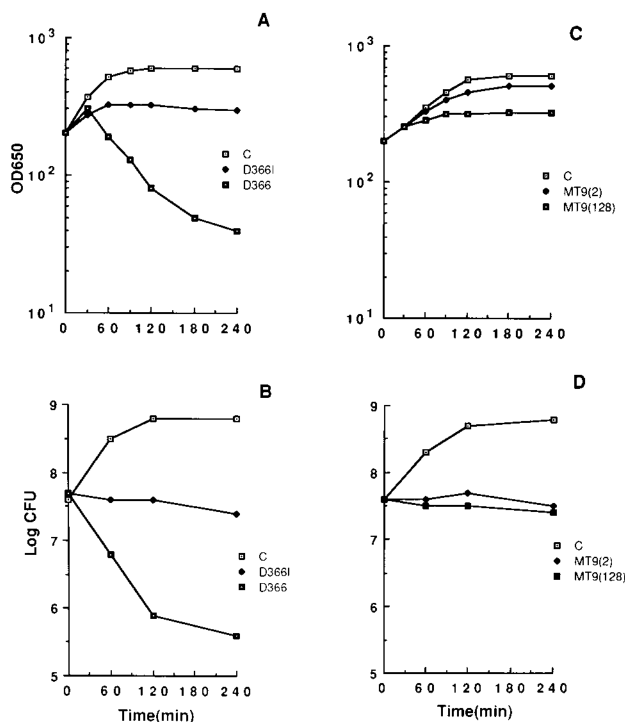

FIG. 1. Lysis curves (A and C) and killing curves (B and D) of the inducible vancomycin-resistant *E. faecium* D366 (A and B) and the constitutive vancomycin-resistant *E. faecium* MT9 (C and D) in the presence of penicillin. Abbreviations for strains: C, control (without antibiotic); D366, *E. faecium* D366 exposed to eight times the MIC (128 μg/ml) of penicillin; D366I, *E. faecium* D366 induced with vancomycin (4 μg/ml) and exposed to penicillin (128 μg/ml); MT9(2), *E. faecium* MT9 exposed to eight times the MIC (2 μg/ml) of penicillin; MT9(128), *E. faecium* MT9 exposed to penicillin (128 μg/ml).

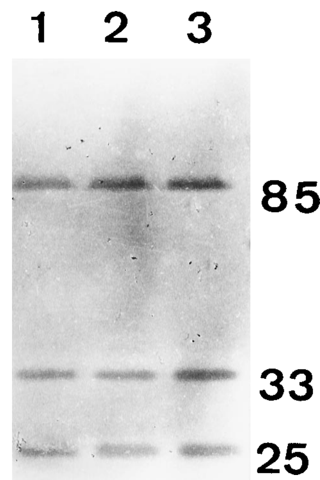

FIG. 2. Visualization of bacteriolytic enzymes. Electrophoresis was performed on an SDS-polyacrylamide gel containing heat-inactivated cells of *E. faecium* D366 to determine the bacteriolytic enzyme profiles of noninduced *E. faecium* D366 (lane 1), *E. faecium* D366 induced with vancomycin (4 μg/ml) (lane 2), and *E. faecium* MT9 (lane 3). Molecular masses (in kilodaltons) are indicated to the right.

These observations suggest that, when vancomycin resistance is expressed, the autolysins are present and still active on the cell wall built in the presence of the new pentadepsipeptide precursor. However, they would appear to be less activated by penicillin in living cells. Nevertheless, one cannot exclude the possibility that an autolysin not detectable by the in vitro cell wall assay might have become inactive.

The possibility that alterations of LTA might be associated with resistance to penicillin-induced lysis was tested. The LTA phosphorus amounted to 24 and 17% of the total phenol-water-extracted phosphorus in strains D366 and MT9, respectively, suggesting a somewhat reduced cellular LTA content in the mutant strain. The analytical data summarized in Table 1 are characteristic of enterococcal LTA (11, 24). The comparison of the LTAs from strains D366 and MT9 shows no obvious difference either in the chain length or in the extent and pattern of glycosylation. The D-alanine ester content in strain MT9, however, was nearly twice as high as that in strain D366NI (Table 1). Interestingly, after induction of strain D366 the amount of D-alanine ester increased 1.8-fold, approaching the value for strain MT9.

The reason for the latter phenomenon might be related to an increased availability of D-alanine due to the observed decrease of the D-Ala–D-Ala dipeptide pool (6), which is linked to the D-D peptidase activity (27) accompanying vancomycin resistance.

LTAs are potent in vitro inhibitors both of autolysins and, when added to growing cultures, of endogenous as well as penicillin-induced cell lysis (8, 9, 22, 29). The antiautolytic in vitro inhibitory effect is dependent on the net negative charge and is gradually reduced by increasing content of positively charged D-alanine ester substituents (16). However, in the aforementioned experiments, LTA was present in the form of micelles with accumulated negative charges on their surfaces (23). When the negative charges were diluted by embedding the LTA into micelles of Triton X-100 or by being subjected to deacylation, yielding the monomeric form (9, 16), LTA lost inhibitory properties. Likewise, monomeric rather than aggregated chains are most likely present in the cytoplasmic membrane, where LTA is surrounded by lipid molecules (19). Evidence for an in vivo role comes from triggering cellular lysis by nisin and Pep5, which was explained by the assumption that these positively charged lantibiotics competitively release the cationic autolysins as a result of their interactions with negatively charged LTA (5). Accordingly, a physiological role of LTA may be the binding of autolysins in the cell wall-membrane complex, but the mechanism which triggers endogenous and penicillin-induced lysis has still to be established. We propose the following hypothesis for *E. faecium*: doubling the alanine ester content observed for LTA when vancomycin re-

TABLE 1. Characterization of LTA purified from *E. faecium* strains[a]

Compound	Ratio to phosphorus found in *E. faecium*:	
	D366NI[a]	MT9
Phosphorus	1.00	1.00
Gro	1.00	1.00
D-Alanine–Gro[b]	0.23	0.47
Glc(α1-2)Gro[b]	0.43	0.38
Glc(α1-2)Glc(α1-2)Gro[b]	0.03	0.05

[a] Values are molar ratios to phosphorus. The chain length, which is the molar ratio of phosphorus to Glc(α1-2)Glc(α1-3)Gro, the deacylated lipid anchor (14), for noninduced strain D366 was 26, and that for MT9 was 24. Glc, D-Glucopyranosyl; Gro, glycerol; D366NI, noninduced strain D366.

[b] Substituted chain glycerol was released with hydrofluoric acid, identified, and quantified as previously described (11).

sistance is expressed would lower the autolysin binding capacity of LTA, which would then affect a step in the pathway that triggers the endogenous as well as the penicillin-induced lytic process. Support for this hypothesis comes from *Bacillus subtilis* mutants that lack the D-alanine ester of LTA and wall teichoic acid (26) and show an increased autolytic rate (29a).

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