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Analysis of Peptidoglycan Precursors in Vancomycin-Resistant Enterococci

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Analysis by high-pressure liquid chromatography of the cytoplasmic peptidoglycan precursors of a high- and a low-level vancomycin-resistant *Enterococcus* spp. was performed before and after induction of resistance. This analysis showed a decrease of the D-Ala-D-Ala and UDP-MurNac-pentapeptide pools, an increase of the UDP-MurNac-tripeptide pool, and the appearance of new UDP-MurNac-containing material. These results lead us to suggest that the vancomycin-induced carboxypeptidase activity cleaves the D-Ala-D-Ala (L. Gutmann, D. Billot-Klein, S. Al-Obeid, I. Klare, S. Francoul, E. Collatz, and J. van Heijenoort, Antimicrob. Agents Chemother. 36:77–80, 1992), which in turn would prevent formation of the normal UDP-MurNac-pentapeptide and thereby of the vancomycin target. The novel UDP-MurNac-containing material is thought to correspond to peptidoglycan precursors which might be synthesized by an alternate pathway (T. D. H. Bugg, G. D. Wright, S. Dutka-Malen, M. Arthur, P. Courvalin, and C. T. Walsh, Biochemistry 30:10408–10415, 1991) and which would be unable to bind vancomycin in glycopeptide-resistant enterococci.

Since 1988 several reports on vancomycin resistance in *Enterococcus faecalis* and *Enterococcus faecium* have appeared (7, 15). Two classes of strains with inducible resistance have been identified: (i) strains with high-level resistance to all glycopeptides, producing the vancomycin-inducible cytoplasmic membrane protein VanA with an apparent molecular mass of 39 kDa, and (ii) strains with moderate levels of resistance to vancomycin but not to teicoplanin, producing the inducible cytoplasmic membrane protein VanB with an apparent molecular mass of 39.5 kDa (16, 21, 22). Analysis of the resistance mechanism has shown that two inducible enzymatic activities are present in the strains of both classes. VanA was identified as a novel D-alanine:D-alanine ligase with altered substrate specificity (5). In conjunction with the α-keto acid dehydrogenase VanH (6), VanA could lead to the formation of a pentapeptide with an altered C-terminal structure lacking affinity for vancomycin. VanB bears some structural similarity to VanA (2) and might have a similar function (5). In addition, in both the VanA- and the VanB-producing strains, the presence of an inducible carboxypeptidase activity, which on the basis of its substrate profile may well be a novel enzyme (12), was demonstrated (1, 12). It was initially hypothesized that this enzyme would cleave the terminal D-alanine of the pentapeptide, thus forming a tetrapeptide unable to bind vancomycin (1). On the basis of these observations (5, 6, 12) one could expect that the cytoplasmic peptidoglycan metabolism would be deeply modified in the induced resistant strain. For this reason, we have undertaken to analyze the pool of the cytoplasmic peptidoglycan precursors from noninduced and induced cells of one strain with a low level and one strain with a high level of vancomycin resistance.

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MATERIALS AND METHODS

Strains and growth conditions. *E. faecium* D366 (VanB producing, moderately resistant to vancomycin, and susceptible to teicoplanin) and *E. faecalis* A256 (VanA producing and highly resistant to both vancomycin and teicoplanin) were previously described (1, 21, 22). DNA from strain A256 hybridized with a *vanA*-derived (9) oligonucleotide (20). Growth was promoted in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C, and turbidity was monitored with a spectrophotometer (Junior III; Perkin-Elmer Corp., Norwalk, Conn.) at 650 nm.

For induction, *E. faecium* D366 and *E. faecalis* A256 were grown overnight in 8 ml of broth in the presence of vancomycin at 4 and 64 μg/ml, respectively, and then diluted into 400 ml of medium containing the same respective concentrations of vancomycin. Induction was verified in fractions of the cultures by searching for the presence of the ca. 39.5- and 39-kDa proteins, respectively, in the cytoplasmic membranes (21, 22).

Chemicals. D-[^{14}C]analyl-D-[^{14}C]alanine (2.2 GBq/mmol) was prepared as previously described (11). Different standard compounds were used: UDP-MurNac-L-Ala-γ-D-Glu-L-Lys, prepared according to the method of Ito et al. (14), was kindly provided by M. Guinand (Laboratoire de Chimie Biologie, Université de Lyon I, Villeurbanne, France); UDP-MurNac-L-Ala-γ-D-Glu-L-Lys-D-Ala-D-Ala was accumulated in *Staphylococcus aureus* by incubation in the presence of vancomycin and extracted as described by Park and Chatterjee (18); UDP-MurNac-tetrapeptide was obtained from UDP-MurNac-pentapeptide in the presence of cytoplasmic membranes of induced *E. faecalis* A256 expressing high carboxypeptidase activity. The reaction was carried out with 5 nmol of UDP-MurNac-pentapeptide and 30 μg of cytoplasmic membranes in 60 μl of sodium phosphate buffer (50 mM, pH 7) for 30 min at 37°C. Vancomycin was kindly provided by Eli Lilly and Co., St. Cloud, France.

Quantification of muramic acid and amino acids. The

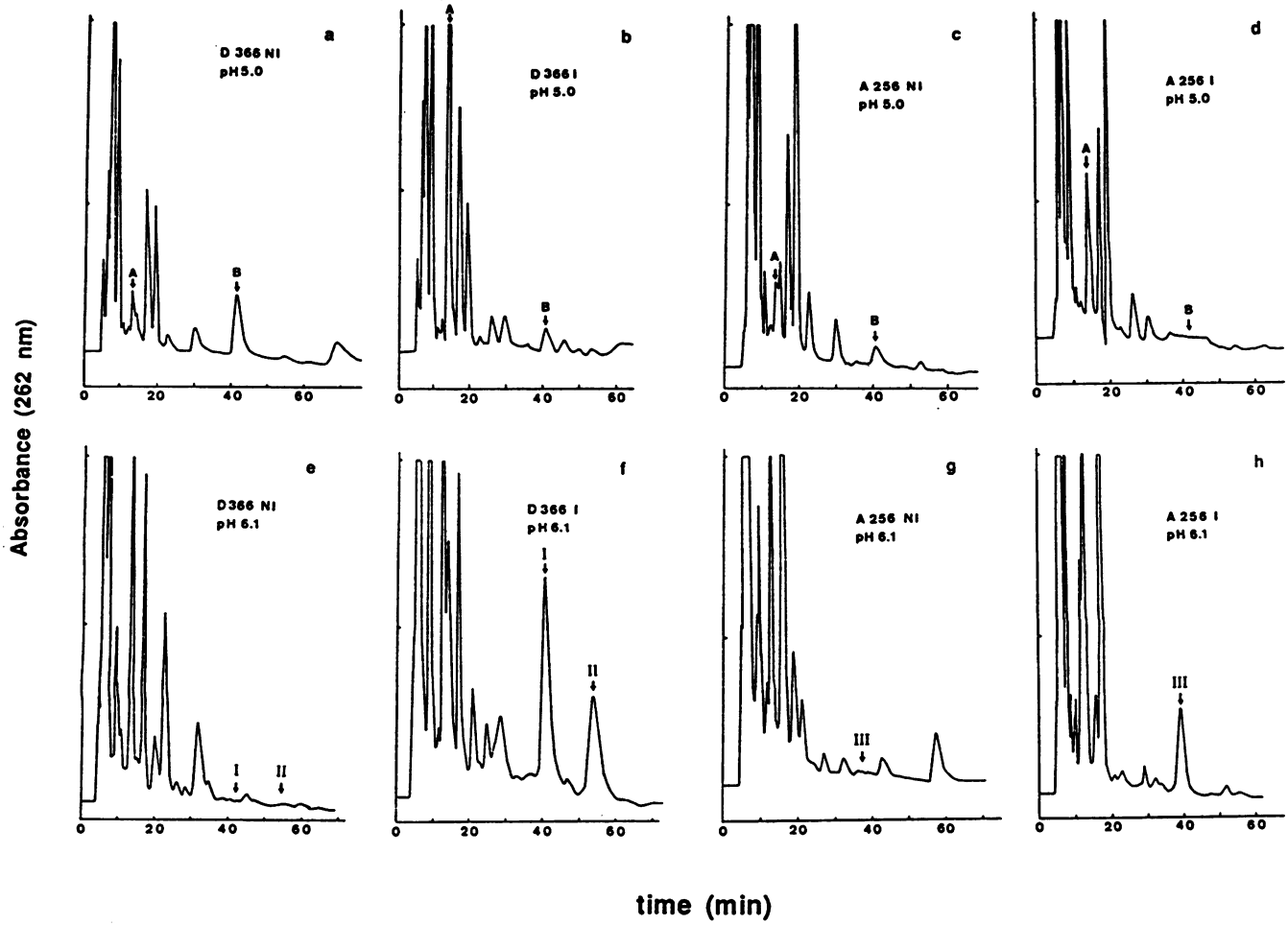

FIG. 1. Separation of UDP-MurNac-peptide precursors by HPLC. A sample of 1/20 of the total extract from 1 liter of culture was applied to a μBondapak C18 column (7.8 by 300 mm). The operating conditions were isocratic elution at room temperature with 0.05 M ammonium formiate at pH 5 (a, b, c, and d) or pH 6.1 (e, f, g, and h). The positions of the UDP-MurNac-peptide precursors are indicated by arrows: A, UDP-MurNac-tripeptide; B, UDP-MurNac-pentapeptide. Peaks I, II, and III (f and h) represent novel UDP-MurNac-precursors. Cytoplasmic precursors of noninduced (NI; a and e) and induced (I; b and f) *E. faecium* D366 and of noninduced (NI; c and g) and induced (I; d and h) *E. faecalis* A256 are shown.

muramic acid and amino acid contents in different high-pressure liquid chromatography (HPLC) fractions were determined with a Biotronik model LC2000 automatic amino acid analyzer after hydrolysis in 6 N HCl at 95°C for 16 h.

Extraction and quantification of peptidoglycan precursors. Extraction of cytoplasmic peptidoglycan precursors was carried out according to the method of Bochner and Ames (4). In brief, 400 ml of an exponential-phase culture at an optical density at 650 nm of 0.4 was centrifuged at 11,000 × *g* and 30°C; 80 ml of ice-cold 1 M formic acid was rapidly added to the pellet (final pH: 2), and nucleotides were extracted for 30 min at 4°C before centrifugation (15,000 × *g* for 20 min, 4°C). The supernatant was lyophilized. Subsequent purification and analytical procedures used for the identification and quantification of the precursors were as previously described (10, 17).

Quantification of D-alanyl-D-alanine. D-Alanyl-D-alanine (D-Ala-D-Ala) was isolated from the supernatants of formic acid-treated cells by a two-step procedure: (i) gel filtration on Sephadex G-25 (fine) and (ii) chromatography on Whatman 3MM filter paper in the solvent system butanol-acetic acid-water (4:1:1). To allow detection and evaluation of recovery, 2,000 Bq of D-[^{14}C]Ala-D-[^{14}C]Ala was added to the initial extracts. D-Ala-D-Ala was quantified with the Biotronik amino acid analyzer in sodium citrate buffer (66 mM, pH 3.95) containing 0.1 M NaCl.

RESULTS AND DISCUSSION

The results of the extractions with formic acid and the quantitative analysis comparing the cytoplasmic precursors, including D-Ala-D-Ala, from noninduced and vancomycin-induced cells, are presented in Fig. 1 and Table 1. With UDP-MurNac-peptide standards, UDP-MurNac-tripeptide and UDP-MurNac-pentapeptide could be located (Fig. 1). After isolation of the corresponding peaks, analysis of the amino acid contents showed that the UV-absorbing material was composed mainly of the respective precursors. In the noninduced strains the UDP-MurNac-pentapeptide pool was approximately five to six times larger than the UDP-MurNac-tripeptide pool. As shown in Fig. 1 and Table 1, a decrease of the UDP-MurNac-pentapeptide pool was ob-

TABLE 1. Pool levels of peptidoglycan precursors in *E. faecium* D366 and *E. faecalis* A256

Strain[a]	Pool level(s) (nmol/liter of culture) of:			
	D-Ala-D-Ala	UDP-MurNac-tripeptide	UDP-MurNac-pentapeptide	UDP-MurNac x[b]
E. faecium D366				
Noninduced	305	40	270	<1
Induced	75	1,125	86	95 (peak I), 108 (peak II)
E. faecalis A256				
Noninduced	570	11	63	<1
Induced	50	114	<1	74 (peak III)

[a] Induced, *E. faecium* D366 and *E. faecalis* A256 induced in the presence of 4 and 64 μg of vancomycin per ml, respectively.
[b] Nanomoles of muramic acid.

served in the induced strains. This decrease was threefold for the strain with a low level of vancomycin resistance, *E. faecium* D366, and more drastic for the strain with a high level of resistance, *E. faecalis* A256, from which no UDP-MurNac-pentapeptide was recovered. The decreases of the UDP-MurNac-pentapeptide pool were associated, respectively, with ca. 4- and 11-fold decreases of the D-Ala-D-Ala pool and ca. 30- and 10-fold increases of that of the UDP-MurNac-tripeptide.

One explanation for the decrease of the D-Ala-D-Ala pool in the resistant strain could be the activity of the vancomycin-inducible carboxypeptidase which is able to cleave the dipeptide in vitro (1, 12), provided that it is active in the cytoplasm. It cannot, however, be excluded that the small D-Ala-D-Ala pool observed in the resistant strains is due to a specific inhibition of the normal ligase, as suggested by Bugg et al. (6).

Since D-Ala-D-Ala is normally added to the UDP-MurNac-tripeptide to yield UDP-MurNac-pentapeptide (8, 19), the decrease of the D-Ala-D-Ala pool could explain the decrease of the UDP-MurNac-pentapeptide pool as well as the increase of the UDP-MurNac-tripeptide pool. Such variations in the precursor pools upon treatment with D-cycloserine, an inhibitor of the normal D-Ala-D-Ala ligase, have been reported previously (13).

Interestingly, in the strain with a high level of resistance, *E. faecalis* A256, in which the carboxypeptidase activity was induced to the higher level (1, 12), the larger decrease of the D-Ala-D-Ala pool occurred, and this was associated with the total disappearance of the UDP-MurNac-pentapeptide pool. This absence of UDP-MurNac-pentapeptide could contribute to the high level of resistance to vancomycin (256 μg/ml), since no peptidoglycan unit with a D-Ala-D-Ala C terminus would be expected to end up in the cell wall and bind vancomycin. In contrast, the low residual level of UDP-MurNac-pentapeptide in the induced *E. faecium* D366 might allow, after its incorporation into the cell wall, binding of vancomycin and thereby explain the low level of vancomycin resistance (32 μg/ml) observed in this strain (22).

We have previously hypothesized (1) that the vancomycin-induced carboxypeptidase activity could cleave the terminal D-alanine of the UDP-MurNac-pentapeptide in vitro, a reaction which should yield UDP-MurNac-tetrapeptide. However, no significant amounts of UDP-MurNac-tetrapeptide, expected to elute between 20 and 24 min, were observed before or after vancomycin induction. This observation is consistant with the induced carboxypeptidase acting preferentially on D-Ala-D-Ala in vivo.

According to the work of Dutka-Malen et al. (9) and Bugg et al. (5), a novel vancomycin-inducible ligase, i.e., VanA, and an α-keto acid dehydrogenase, VanH (3, 6), are present in strains with high levels of resistance (VanB [2] might be a functional equivalent of VanA in strains with low levels of resistance [5]). In vitro, ester bond formation, catalyzed by VanA, between D-alanine and D-hydroxy products of VanH (e.g., D-2-hydroxybutyrate) was observed, as was the subsequent incorporation of the VanA product into the UDP-MurNac-pentapeptide peptidoglycan precursor (6). One would expect that a modified UDP-MurNac-pentapeptide would also be synthesized in vivo and incorporated into peptidoglycan, provided that the enzymatic machinery allows this. Indeed, novel muramic acid-containing material was found in the two induced strains but eluted late in an ill-defined peak at pH 5 (data not shown). Therefore, the precursor extracts of the noninduced and induced cells were examined after elution at pH 6.1 (Fig. 1). Two new peaks (I and II) were found in the extracts of the induced cells of *E. faecium* D366, while the extracts of the induced cells of *E. faecalis* A256 yielded only one new peak (III). After isolation and rechromatography of the material from these peaks, it was shown that peaks I and III eluted with the same retention time. Preliminary analysis of the amino acid and sugar contents of these peaks revealed the predominance of muramic acid, glutamic acid, lysine, and alanine. No significant amounts of phenylalanine or methionine, which had previously been found in mixed dipeptides whose synthesis was preferentially catalyzed in vitro by VanA (5), were observed. Recovery of larger amounts of this material will, however, be necessary to determine the exact compositions and structures of these new muramic acid-containing compounds. If a novel C-terminal D-Ala-X, possibly D-alanyl-D-2-hydroxybutyrate (6), was added to UDP-MurNac-tripeptide in vivo, the fact that this tripeptide was significantly increased in the vancomycin-induced cells could mean that the novel depsipeptide was less readily added than D-Ala-D-Ala.

Considering the currently available data, resistance to vancomycin could be the consequence of the concerted action of an inducible ligase with altered substrate specificity, of a D-specific α-keto dehydrogenase, and of an inducible carboxypeptidase.

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