RESEARCH COMMUNICATION

Peptidoglycan structure of *Enterococcus faecium* expressing vancomycin resistance of the VanB type

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Resistance to glycopeptide antibiotics in enterococci is due to the synthesis of UDP-MurNAc-tetrapeptide-D-lactate (where Mur is muramic acid) replacing the normal UDP-MurNAc-pentapeptide precursor. The peptidoglycan structures of an inducible VanB-type glycopeptide-resistant *Enterococcus faecium*, D366, and its constitutively resistant derivative, MT9, were determined. Using HPLC, 17 mucopeptides were identified and were present regardless of whether resistance was expressed or not. The structures of 15 mucopeptides were determined using MS and

amino acid analysis. The cross-bridge between D-alanine and L-lysine consisted of one asparagine. No monomer pentapeptide or tetrapeptide-D-lactate could be identified. These results obtained with D366 (non-induced) and MT9 indicate that, in the absence of vancomycin, the cell wall synthetic machinery of *E. faecium* can process the lactate-containing precursor as efficiently as the normal pentapeptide. In contrast, the presence of subinhibitory inducing concentrations of vancomycin interfered with the synthesis of oligomers.

INTRODUCTION

Two classes of acquired resistance to the glycopeptide antibiotics, VanA and VanB, have recently appeared in the genus *Enterococcus* and in particular in *E. faecalis* and *E. faecium* [1]. In the VanA class, resistance is generally encoded by plasmids and is inducible by vancomycin and teicoplanin. Resistance is determined by a set of genes in an operon, including *vanA* and *vanH* which encode respectively a ligase and a dehydrogenase, allowing the synthesis of UDP-MurNAc-tetrapeptide-D-lactate (where Mur is muramic acid) which is unable to bind glycopeptides [1–7]. Concomitantly, the pool level of the normal UDP-MurNAc-pentapeptide with its C-terminal D-alanine is decreased [4,8] due to the presence of a DD-peptidase, VanX, which splits the D-alanyl-D-alanine necessary for pentapeptide synthesis [9]. VanY, a membrane-bound carboxypeptidase, is not required for resistance but serves to increase the level of expressed resistance [8,10]. The level of resistance to vancomycin is mainly determined by the relative pool sizes of the new UDP-MurNAc-tetrapeptide-D-lactate precursor and the normal residual UDP-MurNAc-pentapeptide precursor [4,8,10]. Once transported to the cell surface by the lipid intermediate, the precursor is processed by a number of different membrane-bound transpeptidases and carboxypeptidases (penicillin-binding proteins or PBPs).

In the VanB class of glycopeptide-resistant enterococci, the resistance is encoded by large plasmids or the chromosome, is not inducible by teicoplanin and is determined by a set of genes which resemble those of the VanA class, particularly in the case of the ligase [7,11,12]. Using *E. faecium* D366 (*vanB*) [13], either uninduced or induced with vancomycin for expression of resistance, and MT9, a constitutively resistant derivative of D366 [14], we were able to study strains producing varying levels of the

UDP-MurNAc-tetrapeptide-D-lactate precursor. The relative amounts of the new tetrapeptide-D-lactate precursor with respect to the normal pentapeptide precursor in uninduced D366, induced D366 and in MT9 was 0, 60 and 100 % respectively ([8] and D. Billot-Klein, unpublished work). Using a combination of reverse-phase HPLC, MS and amino acid analysis, the aim of this study was to do an in-depth analysis of the peptidoglycan structure of *E. faecium* and a comparative study of this structure built with or without the lactate-containing precursor.

EXPERIMENTAL

Strains and growth conditions

All strains used in this study were routinely cultured on brain heart infusion medium (Difco) at 37 °C. *E. faecium* strain D366 is a clinical isolate expressing an inducible VanB-type resistance [minimal inhibitory concentration (MIC, in µg/ml) of vancomycin was 32 and of teicoplanin 0.5] [13]. Strain MT9 is a derivative of D366, selected on teicoplanin, expressing glycopeptide resistance constitutively (MIC of vancomycin was 128 and of teicoplanin 32) [14].

Preparation of peptidoglycan

Peptidoglycan was extracted from 500 ml of an exponential-phase culture grown up to an A_{650} of 0.4 using a modification of previously described methods [15,16]. To induce vancomycin resistance, D366 was first grown overnight in brain heart infusion medium with vancomycin at 8 µg/ml. It was then back-diluted and grown as described above in the same concentration of vancomycin. The culture was quickly chilled in an ice-bath. Cells

Abbreviations used: PBP, penicillin-binding protein; MIC, minimal inhibitory concentration; Mur, muramic acid; DS, disaccharide GlcNAc–MurNAc; di, dipeptide; tri, tripeptide; tetra, tetrapeptide; Bis, dimeric form; Ter, tetrameric form.

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were harvested at 5000 *g*, and subsequently boiled in 4% (w/v) SDS for 30 min. Cell walls were centrifuged for 30 min at 40 000 *g* and washed six times with water to eliminate SDS. The pellet was treated with Pronase (200 µg/ml) for 16 h at 37 °C in 10 mM Tris/HCl (pH 7.4)/0.02% (w/v) sodium azide. After centrifugation at 40 000 *g* (4 °C) the pellet was treated with trypsin (200 µg/ml) for 16 h at 37 °C in 20 mM potassium phosphate buffer (pH 7.8). The pellet was washed twice with water and treated with lysozyme (200 µg/ml) and mutanolysin (250 µg/ml) for 16 h at 37 °C in 1 ml of 25 mM potassium phosphate buffer (pH 6.5)/10 mM MgCl₂. The enzymes were inactivated by boiling for 3 min. After centrifugation (12 000 *g* for 5 min in an Eppendorf microcentrifuge) the supernatant was stored at -20 °C. We estimated that 90–95% of the enterococcal peptidoglycan was solubilized by this treatment.

Separation of muropeptides

Before separation, samples were mixed with equal volumes of borate buffer (0.5 M, pH 9) and reduced with sodium tetrahydroborate for 15 min at room temperature. The pH of the solutions was adjusted to 4 with orthophosphoric acid (a treatment which was found not to cleave the terminal lactate of the UDP-MurNAc-tetrapeptide-D-lactate precursor). Samples were kept at -20 °C. Separation of the digested cell wall components was performed according to the procedure of Glauner [17], with some modifications. The HPLC system consisted of a Merck L6200 A pump and a L4250 UV detector, a Waters 717 autosampler and a Merck D2500 chromatointegrator.

Samples were applied to an Interchim 250 × 4.6 mm reverse-phase column (ODS-Hypersil, C₁₈, 3 µm) guarded by a Merck Lichrospher (100 RP18, 5 µm) precolumn. The column was eluted at a flow rate of 0.5 ml/min for 10 min with 0.05% (v/v) trifluoroacetic acid in water and subsequently with a 90 min linear acetonitrile gradient (0–20%) in 0.035% trifluoroacetic acid. The column temperature was maintained at 30 °C. The eluted compounds were detected by absorption at 210 nm.

Identification of the peaks

Liquid chromatography/MS was performed using a Hewlett Packard HP1090 Series II liquid chromatograph coupled to either a Finnigan (San Jose, CA, U.S.A.) TSQ700 triple quadrupole mass spectrometer or a Sciex (Thornhill, Ontario, Canada) API-III triple quadrupole mass spectrometer, both equipped with nebulizer-assisted electrospray sources. Data acquisition was performed between 500 and 1900 Da with scan times of the order of 2–3 s. No eluent splitting was used with the Finnigan instrument, while the eluent flow entering the Sciex was split 10:1 so that about 20 µl/min was entering the mass spectrometer. MS/MS was performed on singly and doubly charged protonated molecules using either helium or argon as the collision gas.

The muropeptide structures deduced from the MS of the different samples were confirmed either by fragmentation using an MS/MS system, or chemically, after purification of the peak material by HPLC using the same gradient as before and a Merck 125 × 4 mm column (Lichrospher 100 RP18, 5 µm). The muramitol, glucosamine and amino acid contents of the reduced muropeptides were determined after hydrolysis with 6 M HCl at 95 °C for 16 h using the AccQTag Waters method (Waters Corporation, Milford, MA, U.S.A.). After derivatization, the different components were separated by HPLC and detected with a Waters scanning fluorescence detector 474 and the Waters kit 150 × 3.9 mm column using the gradient recommended by the

manufacturer. Deamidation of muropeptide was obtained by treating the reduced isolated samples at pH 12.5 for 4 h [15]. Comparison of the control and deamidated muropeptides was carried out either by HPLC, using the same gradient conditions as those used for the purification of the muropeptides, or by determination of the mass of the amidated and deamidated peak materials using LC/MS.

RESULTS

Muropeptide composition in *E. faecium* in the absence of expression of vancomycin resistance

The muropeptide composition in *E. faecium* D366 grown in the absence of vancomycin (D366NI) was studied first, since under these conditions vancomycin resistance is not expressed and only the normal UDP-MurNAc-pentapeptide precursor is synthesized [8]. The chromatographic separation of the muropeptides of D366 is presented in Figure 1. As could be expected, the same profile was observed for a vancomycin-susceptible strain *E. faecium* D359 ([18]; results not shown). Using LC/MS, a mass could be detected for the UV-absorbing material in 17 of the peaks. Seven monomeric muropeptides, six dimeric muropeptides

Figure 1 Separation of *E. faecium* cell wall muropeptides by reverse-phase HPLC

Peptidoglycan was obtained from *E. faecium* D366, either non-induced (D366NI) or induced in the presence of vancomycin (8 µg/ml) (D366I) and from the constitutive vancomycin-resistant *E. faecium* MT9 (MT9). Preparation of cell walls was carried out as described in the Materials and methods section. Chemical structures assigned to the muropeptides (labelled with numbers in the chromatogram) are shown in Table 1 and Figure 2.

Table 1 Molecular mass and composition of mucopeptides from *E. faecium* D366 non-induced (NI), or induced (I), by vancomycin, and from the constitutive vancomycin-resistant *E. faecium* MT9 separated by HPLC

The peak numbers correspond to the mucopeptide peaks numbered in Figure 1. Structures correspond to those shown in Figure 2: N, asparagine; (NH₂) indicates the presence of isoGln; (OH) indicates the presence of isoGlu. M is molecular mass as deduced from MS.

Peak number	Structure	M	Strain					
			D366NI	D366I	MT9			
	Monomers		36.5*	72.2	34.4			
1	DS-di(NH ₂)	697	1.5	4.7	3.1			
2	DS-tri(NH ₂)	825	4.9	1.6	3.9			
3	DS-N-tri(OH)	940	6.5	(22.9)†	16.3	(46.8)	8.1	(18.9)
5	DS-N-tri(NH ₂)	939	11.5	19.9	6.9			
4	DS-tetra(NH ₂)	896	2.4	1.3	1.6			
6	DS-N-tetra(OH)	1011	6.1	(12.1)	21.6	(29.7)	8.3	(12.4)
7	DS-N-tetra(NH ₂)	1010	3.6	6.8	2.5			
	Dimers		42.6	23.5	39.8			
8	Bis-DS-tetra(NH ₂)-N-tri(NH ₂)	1819	4.2	—	5.2			
9	Bis-DS-N-tetra(OH)-N-tri(NH ₂)‡	1933	15.1	(35.6)	10.2	(18.0)	16.2	(31.6)
10	Bis-DS-N-tetra(NH ₂)-N-tri(OH)‡	1933	1	—	2.4			
11	Bis-DS-N-tetra(NH ₂)-N-tri(NH ₂)	1932	15.3	7.8	7.6			
12	Bis-DS-N-tetra(OH)-N-tetra(OH)	2005	1.6	(7.0)	0.9	(5.5)	1.2	(8.8)
13	Bis-DS-N-tetra(NH ₂)-N-tetra(OH)‡	2004	5.4	4.6	7.6			
	Trimers		20.9	4.3	25.8			
14	Ter-DS-N-tetra(NH ₂)-N-tetra(NH ₂)-N-t ri(OH)‡	2925	13.4	(17.8)	4.3	(4.3)	12.9	(18.1)
15	Ter-DS-N-tetra(NH ₂)-N-tetra(NH ₂)-N-t ri(NH ₂)	2924	4.4	—	5.2			
16/17	Ter-DS-N-§	3024	2	—	7.1		(7.1)	

* The values are presented as a percentage of the sum of all peaks presented in the Table and are the average of two or three experiments.

† Numbers in parentheses represent subtotals within each subclass.

‡ The assignment of the amide and the hydroxyl functions to either peptide stem is arbitrary.

§ Precise structure unknown.

and four trimeric mucopeptides were identified (Table 1 and Figure 2). The structure was generally deduced from the molecular mass obtained by MS and confirmed for some relevant mucopeptides by amino acid analysis. Mur, GlcN, Ala, Glx, Lys and Asn were found in ratios of: 1:1:1.3:0.7:0.9:1.3 for mucopeptide present in peak 3; 1:1:1.9:0.9:0.8:0.9 for mucopeptide present in peak 6; and 2:2:3.5:2.2:1.7:1.7 for mucopeptide present in peak 9. In the case of the structure present in peak 3, MS/MS (results not shown) confirmed that the amino acid sequence was indeed GlcNAc-MurNAc-Ala-Gln-(Asn)-Lys. The molecules contained in peaks 16 and 17 were of identical molecular mass, 3024 Da, consistent with a trimeric mucopeptide, but no precise structure could be deduced. No peaks corresponding to GlcNAc-MurNAc pentapeptide with a C-terminal D-alanine, or its Asn-substituted form, could be identified.

Deamidation of mucopeptides 5 (DS-tri), 7 (DS-tetra), 11 (Bis-DS-tetra-tri) and 15 (Ter-DS-tetra-tetra-tri) resulted in products which, during HPLC, co-migrated with peaks 3, 6, 9 and 14 respectively. This suggested that the difference in molecular mass was exclusively due to substitution of NH₂ by OH groups. Examination by MS of the products obtained after deamidation of mucopeptides 7 and 6 (Table 1, Figure 3) showed that from mucopeptide 7 (molecular mass 1010 Da) two products were obtained, with molecular masses of 1011 and 1012 Da, while from mucopeptide 6 (molecular mass 1011 Da) only one product was obtained, with a molecular mass of 1012 Da. As shown in Figure 3, this suggested that deamidation of the glutamine and asparagine residues could occur successively in mucopeptide 7. In mucopeptide 6, only deamidation of asparagine would occur if one assumes, as suggested previously [19] and by the mass

found for many of the peaks in this study, that only asparagine, and not aspartic acid, is linked to the ϵ -amino group of lysine in the native mucopeptide. Finally, the results of MS, deamidation, and amino acid analysis taken together, show that Ala-Asn-Lys [19] forms the cross-bridge between both the dimers and trimers (Figure 2).

Mucopeptide composition after expression of vancomycin resistance

The mucopeptide composition of *E. faecium* MT9, which expresses resistance constitutively, was compared with that of the non-induced D366 (Figure 1, Table 1). The HPLC profile of the digested peptidoglycan of strain MT9 was very similar to that of D366. The molecular masses of the 17 identified mucopeptides were identical with those of D366. Only variations in the relative amounts of some amidated and non-amidated mucopeptides were found (Table 1, Figure 1), with an apparent decrease of some amidated mucopeptides in MT9 (e.g. compare peaks 5, 7 and 11 between MT9 and D366NI). No additional peaks corresponding to new, defined structures could be identified and, in particular, no structure corresponding to a monomer mucopeptide-D-lactate unit was observed. When the mucopeptide composition of *E. faecium* D366, in which expression of vancomycin resistance had been induced with 8 µg/ml of vancomycin (D366I), was examined, while again the HPLC profile was very similar to that of D366NI, the overall quantity of dimers and trimers was significantly lower (Figure 1). The oligomer/monomer ratio was half of that observed for D366NI and MT9 (Table 1). Again, as for MT9, no new structure was identified.

Figure 2 Detail of the structures present in peaks 1–15 of Figure 1 and Table 1

Abbreviations: G, GlcNAc; M, MurNAc; iGln, isoGln; iGlx, isoGlx.

Figure 3 Proposed structures for the deamidated products: 7, [DS-tetra-(NH₂)-N]; and 6, [DS-tetra-(OH)-N]

Structures were derived from chemical and mass spectrometric analyses [assignment of the amide function (NH₂) in compound 6 is arbitrary]. Abbreviations: M, molecular mass; New, new structure obtained after deamidation of products 7 and 6.

DISCUSSION

In the *E. faecium* strain D366, in the absence of expression of vancomycin resistance, analysis of the peptidoglycan structure showed that it is composed of an acceptor mucopeptide in which the lysine residue is substituted at its ϵ -amino group by an asparagine directly linked to the D-Ala-4 of the donor, in agreement with earlier data [19]. As previously shown for *Gaffkya*

homari [15], the predominant form of the amino acid in position 2 was an α -amidated γ -D-glutamyl residue. Although UDP-MurNAc-pentapeptide is the final cytoplasmic precursor in this species [8], no muropentapeptide was found and one third of the monomers were DS-tetrapeptides, suggesting that carboxypeptidase(s) are present. Carboxypeptidase activities have been found in the cytoplasmic membranes of non-induced D366 and non-Van *E. faecium* strains [20]. At least one of the low-molecular-mass PBPs could play this role, as demonstrated for PBP6 of *Enterococcus hirae* [21]. The large quantity of murotripeptide (63%) present among the monomers would suggest that either an LD-carboxypeptidase exists in *E. faecium* or that, as previously suggested for *E. coli* [22], there is direct transport of the tripeptide precursor by the lipid intermediate. One cannot exclude that these murotri- and murotetra-peptides could possibly serve as acceptors, as shown by the structure of different oligomers present in the cell wall.

In the peptidoglycan of *E. faecium*, oligomers were more abundant than monomers, and amidated monomers and oligomers were predominant. This resembles the situation in *Gaffkya homari* where it has been shown that amidated monomers are preferentially utilized as acceptors [15,23].

For MT9, which constitutively expressed resistance to vancomycin when grown in the absence of vancomycin, the overall structure of peptidoglycan was very similar to that of the non-induced D366. In addition, analysis of cytoplasmic peptidoglycan precursors in MT9 demonstrated that UDP-MurNAc-tetrapeptide-D-lactate was synthesized and that the normal pentapeptide precursor was not detectable (D. Billot-Klein,

unpublished work). Therefore, in MT9, one would expect a depsipeptide monomer unit, DS-MurNAc-Ala-D-iGlx-(ϵ -amino-Asn)-Lys-D-Ala-D-Lac, to be translocated and to be easily processed by the different enzymes (transpeptidases and carboxypeptidases) involved in the polymerization of the cell wall. In this regard it is worth noting that the PBP profile of MT9 was identical with that of D366 [14].

Neither the peptidoglycan of uninduced D366 nor that of MT9 contained detectable muropentapeptide or murotetrapeptide-D-lactate respectively. This would again suggest that carboxypeptidases may act efficiently both on the muropentapeptide and murotetrapeptide-lactate monomers, and may therefore explain the high quantities of murotetrapeptide found. A previous study *in vitro* indicated that bacterial DD-carboxypeptidases from other species cleave the depsipeptide D-Ala-D-Lac linkage as efficiently or even more efficiently than the D-Ala-D-Ala linkage [24]. In the particular case of MT9, the constitutively expressed VanY carboxypeptidase [14,20] may play an additional role in the processing of the murotetrapeptide-D-lactate.

When vancomycin resistance in D366 was induced in the presence of vancomycin, the major impact was a significant decrease in oligomers, which is in apparent contrast with what was observed for MT9 grown in the absence of the antibiotic. Interestingly, we have shown in D366, that after induction of vancomycin resistance, a residual quantity of the normal UDP-MurNAc-pentapeptide precursor (30% of the non-induced strain) was present during the production of the new UDP-MurNAc-tetrapeptide-D-lactate precursor [4,8]. It is therefore possible that the presence of this pentapeptide precursor would allow some specific binding of vancomycin, thereby impairing the synthesis of oligomers. Another hypothesis would be that non-specific binding of vancomycin [25] could interfere with the synthesis of oligomers. When MT9 was subjected to the same growth conditions (8 μ g of vancomycin/ml) as were used to induce expression of *vanB* in D366, an oligomer/monomer ratio intermediate between that of MT9 grown without vancomycin and that of D366I was observed (results not shown). Since no normal pentapeptide precursor was found in the cytoplasm of MT9, these observations would favour the latter hypothesis for MT9, but do not exclude the first hypothesis for D366. Therefore, the presence of vancomycin at 8 μ g/ml (four times the MIC for a vancomycin-susceptible *E. faecium* which has not acquired the resistance) decreases the synthesis of oligomers in both D366 and MT9, without affecting the growth rate or the synthesis of monomers. This situation resembles that found in methicillin-resistant *Staphylococcus aureus* grown in the presence of high quantities of methicillin where all PBPs are saturated except PBP2A [16] and where, in spite of a complete absence of oligomers, the strain grows apparently normally.

In conclusion, we have described the structure of *E. faecium* peptidoglycan and shown that constitutive expression of vanco-

mycin resistance of the VanB type in the absence of vancomycin does not substantially affect its composition, even though a new peptidoglycan precursor is synthesized. Thus, once exported, this precursor seems to be perfectly well recognized by the cell wall synthetic machinery.

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