RESEARCH COMMUNICATION

Analysis of peptidoglycan precursors in vancomycin-resistant *Enterococcus gallinarum* BM4174

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Vancomycin resistance in enterococci is an increasing clinical problem, and several phenotypes have been identified. We demonstrate here that the resistance mechanism in the constitutively vancomycin-resistant *Enterococcus gallinarum* BM4174 involves an altered pathway of peptidoglycan synthesis and hydrolysis of the normal precursors in the vancomycin-sensitive pathway. A ligase encoded by the *vanC* gene catalyses synthesis of D-Ala-D-Ser and substitutes this dipeptide for D-Ala-D-Ala in peptidoglycan precursors. It is presumed that this substitution lowers the affinity of vancomycin for its target site. Destruction of D-Ala-D-Ala (D,D-peptidase activity) and of UDP-MurNAc-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala by removal of the terminal D-Ala residue (D,D-carboxypeptidase activity) ensures that the normal vancomycin-sensitive pathway of peptidoglycan synthesis cannot function in the resistant strain.

INTRODUCTION

High-level resistance to vancomycin and other glycopeptide antibiotics in *Enterococcus faecium* and *E. faecalis* with the VanA phenotype results from changes in the peptidoglycan-biosynthetic pathway (Arthur and Courvalin, 1993). Three enzymes are required for resistance, VanA and VanH for synthesis of D-Ala-D-lactate (Arthur et al., 1993), which is incorporated into peptidoglycan precursors in place of D-Ala-D-Ala (Messer and Reynolds, 1992; Allen et al., 1992; Handwerger et al., 1992), and VanX, a dipeptidase which hydrolyses D-Ala-D-Ala (P. E. Reynolds, F. Depardieu, S. Dutka-Malen, M. Arthur and P. Courvalin, unpublished work), thus decreasing production of the normal peptidoglycan precursor UDP-MurNAc-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala (UDP-MurNAc-pentapeptide[Ala]). A fourth enzyme, the VanY D,D-carboxypeptidase, contributes to glycopeptide resistance by removing D-Ala residues from peptidoglycan precursors (Gutmann et al., 1992; M. Arthur, F. Depardieu, H. A. Snaith, P. E. Reynolds and P. Courvalin, unpublished work).

Two other species of enterococci which possess the VanC phenotype, *E. gallinarum* and *E. casseliflavus*, express constitutive resistance to low levels of vancomycin, but remain sensitive to teicoplanin (Dutka-Malen et al., 1990; Vincent et al., 1991, 1992). The genes encoding resistance are probably chromosomally located, and one of these, *vanC*, has been identified in *E. gallinarum* and its sequence has been determined (Dutka-Malen et al., 1992). The *vanC* gene, specific for *E. gallinarum* (Leclercq et al., 1992), is necessary for expression of vancomycin resistance (Dutka-Malen et al., 1992) and encodes a protein (VanC) which is related to VanA and to D-Ala-D-Ala ligases of *Escherichia coli* (Dutka-Malen et al., 1992). Insertional inactivation of vanC in *E. gallinarum* BM4174 led to the production of the vancomycin-susceptible strain BM4175, indicating that *E. gallinarum* encodes at least one other ligase which presumably catalyses the synthesis of D-Ala-D-Ala (Dutka-Malen et al., 1992). Resistance was not restored by transcomplementation with the cloned *vanA* gene, suggesting that VanC does not catalyse the synthesis of D-Ala-D-lactate (Dutka-Malen et al., 1992).

In this paper we compare the peptidoglycan precursors synthesized in the vancomycin-resistant *E. gallinarum* BM4174 and its vancomycin-susceptible derivative BM4175 (Dutka-Malen et al., 1992) and identify enzyme activities that could be involved in controlling the host glycopeptide-susceptible pathway of peptidoglycan synthesis.

EXPERIMENTAL

Strains and growth conditions

E. gallinarum BM4174, constitutively resistant to low levels of vancomycin, and BM4175, in which plasmid pAT217 containing the erythromycin-resistance gene *erm* had been integrated into *vanC* (Dutka-Malen et al., 1992), were grown with or without vancomycin (2 μg/ml) in brain/heart infusion (BHI) broth supplemented with glucose (1 %, w/v) or on BHI agar at 37 °C. Erythromycin (10 μg/ml) was added to the media for BM4175 to maintain the insert in the *vanC* gene.

Analysis of peptidoglycan precursors

Cultures of *E. gallinarum* (1.25 litres) were grown to mid-exponential phase (A_{600} = 1), ramoplanin (10 μg/ml) was added and incubation continued for 0.66 mean generation time. Bacteria were harvested by centrifugation at 12000 *g* for 2 min at 4 °C, and low-molecular-mass components in the cytoplasm were extracted with 8 % trichloroacetic acid for 15 min at 4 °C as

Abbreviations used: UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-MurNAc, UDP-*N*-acetylmuramic acid; UDP-MurNAc-tripeptide (UDP-MurNAc-3-peptide), UDP-*N*-acetylmuramyl-L-Ala-D-isoGlu-L-Lys; UDP-MurNAc-tetrapeptide (UDP-MurNAc-4-peptide), UDP-*N*-acetylmuramyl-L-Ala-D-isoGlu-L-Lys-D-Ala; UDP-MurNAc-pentapeptide[Ala] (UDP-MurNAc-5-peptide[Ala]), UDP-*N*-acetylmuramyl-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala; UDP-MurNAc-pentapeptide[Ser] (UDP-MurNAc-5-peptide[Ser]), UDP-*N*-acetylmuramyl-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ser; UDP-MurNAc-6-peptide, UDP-*N*-acetylmuramyl-L-Ala-D-isoGlu-L-Lys(D-Asp)-L-Ala-L-Ala; BHI, brain/heart infusion.

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described by Messer and Reynolds (1992). Peptidoglycan precursors were separated by ion-exchange chromatography on Dowex 1 (Cl^- form), by a linear chloride gradient (0–0.3 M NaCl in 0.01 M HCl) and desalted by gel filtration on Sephadex G-10. The precursors in the different u.v.-absorbing peaks that reacted positively for *N*-acetylhexosamine (Strominger, 1957) were identified and assayed in four ways (Messer and Reynolds, 1992): (i) automated amino acid analysis of samples hydrolysed in 6 M HCl for 4 and 24 h; (ii) determination of amino acids released from UDP-MurNAc-pentapeptide derivatives by the D,D-carboxypeptidase from *Actinomadura* R39; (iii) mass determination by positive-ion electrospray m.s.; and (iv) h.p.l.c. on a C_{18} reverse-phase column (Spherisorb-5 μm, 220 mm × 4.6 mm) eluted with 0.05 M ammonium acetate, pH 5.03, containing a gradient of 0–2 % methanol in 0.05 M ammonium acetate applied between 10 and 20 min.

Preparation of enzyme extracts from *E. gallinarum*

Strains were grown in BHI/glucose (100 ml) to a turbidity of 1.0 at 600 nm. Bacteria were harvested by centrifugation (30000 *g* for 30 s at 4 °C), washed in buffer A (50 mM Tris/HCl, pH 7.2) and resuspended in 12 ml of the same buffer. The bacteria were lysed by treatment with lysozyme (200 μg/ml) and M1 muramidase (30 μg/ml) at 37 °C. When lysis was almost complete (15 min), $MgCl_2$ (5 mM) and DNAase (20 μg/ml) were added and incubation was continued for 5 min at 37 °C. The extracts were centrifuged (48000 *g* for 20 min at 4 °C) and the supernatant was collected (cytoplasmic fraction). The pellet was washed in buffer A and resuspended in the same buffer at 5 mg of protein/ml (membrane fraction).

Assay for D,D-peptidase and D,D-carboxypeptidase

The routine assay contained, in a final volume of 30 μl, 75 mM Tris/HCl, pH 7.5, 6 mM $MgCl_2$, 1.66 mM substrate (UDP-MurNAc-pentapeptide[Ala] for D,D-carboxypeptidase and 1.6 mM D-Ala-D-Ala for D,D-peptidase) and enzyme preparation, diluted to ensure that less than 20 % of the substrate was hydrolysed in a 30 min incubation at 37 °C. Benzylpenicillin (250 μg/ml) was included in duplicate incubations to investigate its effect on hydrolysis. D-Ala released was assayed by using D-amino acid oxidase (Messer and Reynolds, 1992). D-Serine was assayed by automated amino acid analysis.

RESULTS

Peptidoglycan precursors in *E. gallinarum* BM4174 and BM4175

Ion-exchange chromatography of the extract from the constitutive vancomycin-resistant *E. gallinarum* BM4174 that had been incubated in the presence of ramoplanin to inhibit peptidoglycan synthesis gave rise to three peaks of *N*-acetylhexosamine-containing material (Figure 1a). Analysis of Peak 1 indicated that two compounds had co-eluted at a salt concentration of 0.12 M, and these were identified as UDP-MurNAc-L-Ala-D-isoGlu-L-Lys-D-Ala (UDP-MurNAc-tetrapeptide) and UDP-MurNAc-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ser (UDP-MurNAc-pentapeptide-[Ser]) in the approximate ratio 3:1, based on h.p.l.c. (Figure 2a), automated amino acid analysis of the hydrolysed precursors, the removal of D-serine with the D,D-carboxypeptidase from *Actinomadura* R39, and m.s. (Table 1). The ratio of the two cell-wall precursors was unchanged in cultures that had been pregrown in a low concentration of vancomycin (2 μg/ml) before treatment with ramoplanin (results not shown). The remaining two peaks

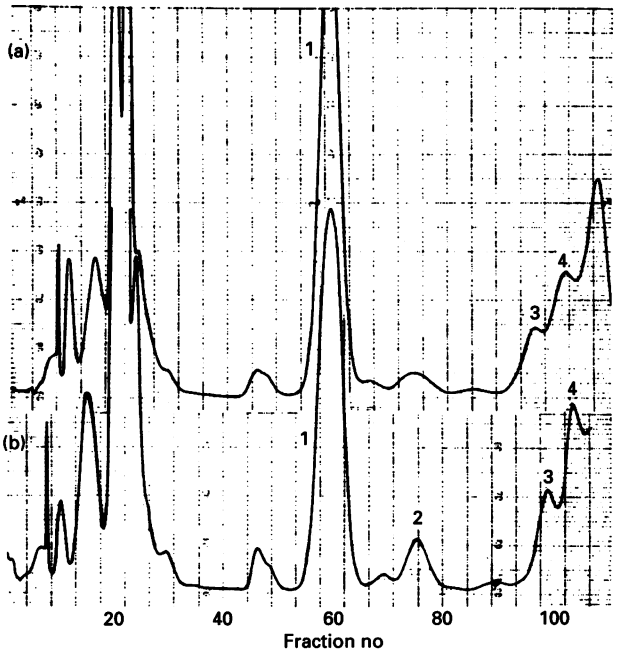

Figure 1 Ion-exchange chromatography on Dowex 1 (Cl^- form) of cytoplasmic constituents, including peptidoglycan precursors, extracted from *E. gallinarum* BM4174 (a) and BM4175 (b), that had been incubated with ramoplanin to inhibit peptidoglycan synthesis

The column effluent was monitored at 252 nm to reveal nucleotides. The peaks containing material reacting positively for *N*-acetylhexosamine are numbered: analysis of the components in these peaks is given in Table 1.

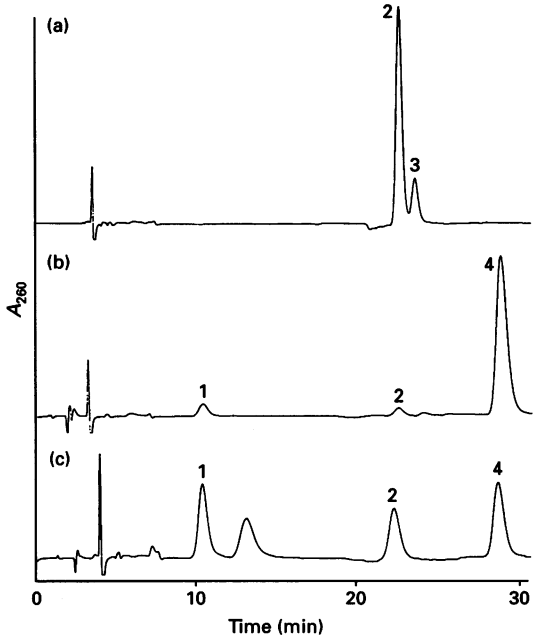

Figure 2 Separation by h.p.l.c. (Spherisorb-5 μm, 220 mm × 4.6 mm) of peptidoglycan precursors present in Peak 1 (Figure 1a) of *E. gallinarum* BM4174 (a) and Peak 1 (Figure 1b) of *E. gallinarum* BM4175 (b)

The precursors were desalted by gel filtration on Sephadex G-10 before application to the C_{18} reverse-phase column. The peaks were identified (Table 1) by reference to the elution profile of standards (**c**): peak 1, UDP-MurNAc-tripeptide; peak 2, UDP-MurNAc-tetrapeptide; peak 3, UDP-MurNAc-pentapeptide[Ala]. The unmarked peak in (**c**) contained UDP-MurNAc-L-Ala-D-isoGlu-*meso*-diaminopimelic acid-D-Ala-D-Ala.

Table 1 Peptidoglycan precursors in *E. gallinarum* BM4174 and BM4175

–, No amino acid detected.

Strain	Precursor in 1.25 litres (μmol)	Ion-exchange peak no.	Precursor (μmol)	Mass*	Amino acid released by R39 D,D-carboxypeptidase† Amino acid	mol (%)	Amino acid proportions† (Asp:Ser:Glu:Ala:Lys)	Identification	Percentage in peak‡
BM4174	21.3	1	14.5	(A) 1079.5	Serine	25	0:0.26:1.02:1.95:[1.0]	UDP-MurNAc-4-peptide	76
				(B) 1167.6				UDP-MurNAc-5-peptide[Ser]	24
		3	2.3		–			UDP-GlcNAc	100
		4	3.3		–			UDP-MurNAc	100
BM4175	21.0	1	13.1	1150.2	D-Alanine	90	0:0:1.03:2.90:[1.0]	UDP-MurNAc-5-peptide[Ala]	91
								UDP-MurNAc-4-peptide	3
								UDP-MurNAc-3-peptide	6
		2	1.8	1265	D-Alanine	100	1.03:0:1.12:3.01:[1.0]	UDP-MurNAc-6-peptide[Ala] (Lys-Asp)	100
		3	2.5		–			UDP-GlcNAc	100
		4	3.4		–			UDP-MurNAc	100

* Determined by positive-ion electrospray m.s.
† Determined by automated amino acid analysis.
‡ Determined by h.p.l.c. (ion-exchange Peak I) or automated amino acid analysis (Peaks 2–4).

of cell-wall precursors (Figure 1a) contained UDP-GlcNAc (Peak 3) and UDP-MurNAc (Peak 4).

Ion-exchange chromatography of the extract of BM4175 (Figure 1b) resulted in a similar pattern of peaks as had been obtained with BM4174, although identification of certain of the precursors led to different results. Peak 1 contained mainly (91%) UDP-MurNAc-pentapeptide[Ala], with small amounts of UDP-MurNAc-tetrapeptide (3%) and UDP-MurNAc-tripeptide (6%) as revealed by h.p.l.c. (Figure 2b), and the small Peak 2 (Figure 1b) contained UDP-MurNAc-L-Ala-D-isoGlu-L-Lys(D-Asp)-D-Ala-D-Ala (Table 1). The Lys–Asp peptide bond was particularly resistant to acid hydrolysis: only 45% of these bonds were hydrolysed with 6 M HCl at 105 °C for 4 h, and complete hydrolysis required treatment for 30–36 h. The identities of the compounds were confirmed by h.p.l.c. analysis using authentic standards of UDP-MurNAc-tripeptide, UDP-MurNAc-tetrapeptide and UDP-MurNAc-pentapeptide[Ala], which were eluted at 10.4, 22.5 and 28.6 min respectively (Figure 2c). Under the same conditions UDP-MurNAc-pentapeptide[Ser] was eluted at 23.8 min. Although *E. gallinarum* BM4174 is constitutively resistant to vancomycin, growth of the organism in BHI/glucose containing a sub-inhibitory concentration of the antibiotic (2 μg/ml), but without the addition of ramoplanin, also resulted in the accumulation of the cell-wall precursors UDP-MurNAc-tetrapeptide and UDP-MurNAc-pentapeptide-[Ser]. This indicates that peptidoglycan synthesis had been partially inhibited at sub-inhibitory concentrations of the antibiotic, although the amounts of the precursors that accumulated were much lower than those obtained after inhibition of peptidoglycan synthesis with ramoplanin.

D,D-Peptidase and D,D-carboxypeptidase activities of *E. gallinarum* BM4174 and BM4175

Glycopeptide-resistant enterococci with the VanA phenotype possess separate enzymes that hydrolyse D-Ala-D-Ala (P. E. Reynolds, F. Depardieu, S. Dutka-Malen, M. Arthur and P. Courvalin, unpublished work) and remove the terminal residue from UDP-MurNAc-pentapeptide (M. Arthur, F. Depardieu, H. A. Snaith, P. E. Reynolds and P. Courvalin, unpublished work). These activities ensure that the D-Ala-D-Ala-dependent vancomycin-susceptible pathway of peptidoglycan synthesis encoded by host genes is effectively eliminated. *E. gallinarum* BM4175 synthesized the normal peptidoglycan precursor, UDP-MurNAc-pentapeptide[Ala]: therefore, it seemed likely that *E. gallinarum* BM4174 would possess enzymes to limit the amounts of D-Ala-D-Ala and/or UDP-MurNAc-pentapeptide present in the cytoplasm. Disrupted cells of BM4174, whether grown in the presence or absence of low concentrations of vancomycin, contained both enzyme activities, which were present mainly in the cytoplasm (Table 2). Extracts of *E. gallinarum* BM4175 contained very low levels of activity against either substrate, indicating that inactivation of the *vanC* gene had also prevented expression of the genes encoding the D,D-peptidase and D,D-carboxypeptidase activities (Table 2). The residual low level of D,D-carboxypeptidase activity present in membrane preparations from this strain was inhibited by benzylpenicillin, indicating that it was probably catalysed by a low-molecular-mass penicillin-binding protein.

The activity of the cytoplasmic extract of *E. gallinarum* BM4174 against UDP-MurNAc-pentapeptide[Ser] was at most 2–3% of the activity against UDP-MurNAc-pentapeptide[Ala]. Therefore the D,D-carboxypeptidase removes the cell-wall precursor involved in the vancomycin-susceptible pathway of peptidoglycan synthesis, but has little effect on the resistant pathway.

DISCUSSION

Vancomycin and teicoplanin bind to the acyl-D-Ala-D-Ala termini of peptidoglycan precursors as they are extruded through the cytoplasmic membrane, and inhibit the polymerization reaction. Replacement of the terminal D-Ala by D-lactate in the

Table 2 D,D-Carboxypeptidase and D,D-peptidase activities of *E. gallinarum* BM4174 and BM4175

		D,D-Carboxypeptidase activity (UDP.MurNAc-5-peptide[Ala])			D,D-Peptidase activity (D-Ala-D-Ala)		
Strain	Fraction	C-terminal residue hydrolysed (nmol/min per mg of bacteria)	% of total	Inhibition by penicillin* (%)	C-terminal residue hydrolysed (nmol/min per mg of bacteria)	% of total	Inhibition by penicillin* (%)
BM4174	Membrane	0.67	8	38	0.69	3	2
	Cytoplasm	7.4	92	4	19.9	97	3
BM4175	Membrane	0.68	66	77	0.08	22	0
	Cytoplasm	0.35	34	6	0.28	78	0

* Benzylpenicillin (250 μg/ml).

precursors of high-level-resistant VanA-type (Messer and Reynolds, 1992) or VanB-type (Evers et al., 1994) enterococci drastically decreases the affinity of glycopeptide antibiotics for the precursors. *E. gallinarum* is constitutively resistant to vancomycin, but remains susceptible to teicoplanin: consequently any structural change in cell-wall precursors is unlikely to be as substantially different as the change from a peptide bond to an ester bond in VanA and VanB strains, a change which eliminates one of the five hydrogen bonds involved in binding of glycopeptides to their target site (Bugg et al., 1991). The replacement of a D-alanine residue by D-serine should not affect the number of hydrogen bonds that can be formed between vancomycin and the altered precursor, but the binding affinity will be altered. The binding pocket in vancomycin is very specific (Barna and Williams, 1984), and the affinity for the target is greatest when the R group of the terminal residue is a methyl group (Nieto and Perkins, 1971). The presence of the hydroxymethyl group in serine is likely to weaken the binding of vancomycin, and consequently this would lead to an increase in the MIC (minimum growth-inhibitory concentration) of vancomycin. *E. gallinarum* BM4174 remains susceptible to teicoplanin, implying that this glycopeptide has a less restrictive binding site in relation to the terminal residue.

E. gallinarum BM4175, in which the *vanC* gene has been insertionally inactivated, is susceptible to vancomycin: it has the normal D-Ala-D-Ala-dependent pathway of wall synthesis, supporting the proposal that a second ligase is functional, as suggested previously (Dutka-Malen et al., 1992) and does not display D,D-dipeptidase or D,D-carboxypeptidase activity in the cytoplasm. In VanA strains in which resistance is encoded by the Tn*1546* transposon, the *vanX* (encoding the dipeptidase) and *vanY* (encoding the D,D-carboxypeptidase) genes are situated downstream from *vanA*, which encodes the altered ligase (Arthur et al., 1993). Therefore, a similar arrangement of the genes may occur in *E. gallinarum* BM4174, although it has not yet been shown whether one or two genes and proteins are involved in the control of the host pathway.

The mechanism of glycopeptide resistance in *E. gallinarum* BM4174 is different from that in *E. faecium* and *E. faecalis*, with VanA and VanB phenotypes, though it relies on the same principle. A new ligase, VanC, with presumed altered specificity for the amino acid at the C-terminus of the dipeptide product, catalyses synthesis of D-Ala-D-Ser, which is added to UDP-MurNAc-tripeptide, giving rise to UDP-MurNAc-pentapeptide[Ser]. Synthesis of the normal precursor UDP-MurNAc-pentapeptide[Ala] is suppressed by hydrolysis of D-Ala-D-Ala and by removal of the terminal D-Ala residue from UDP-MurNAc-pentapeptide[Ala]. The hydrolytic product, UDP-MurNAc-tetrapeptide, may be able to function as a cell-wall precursor in terms of the polymerization reaction, but would be unable to function as the donor substrate in the transpeptidation reaction which cross-links newly-synthesized peptidoglycan to the mature cell wall. It is believed that replacement of the normal precursor by UDP-MurNAc-pentapeptide[Ser], which does not appear to be a substrate for the D,D-carboxypeptidase/D,D-peptidase system of *E. gallinarum*, alters the affinity of vancomycin for the substrate and results in an elevation of the vancomycin MIC.

Note added in proof (received 13 May 1994)

After this paper was submitted, the presence in *E. gallinarum* of a peptidoglycan precursor terminating in serine was reported by Billot-Klein et al. (1994).

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