

Peptidoglycan Cross-Linking in Glycopeptide-Resistant *Actinomycetales*

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Synthesis of peptidoglycan precursors ending in D-lactate (D-Lac) is thought to be responsible for glycopeptide resistance in members of the order *Actinomycetales* that produce these drugs and in related soil bacteria. More recently, the peptidoglycan of several members of the order *Actinomycetales* was shown to be cross-linked by L,D-transpeptidases that use tetrapeptide acyl donors devoid of the target of glycopeptides. To evaluate the contribution of these resistance mechanisms, we have determined the peptidoglycan structure of *Streptomyces coelicolor* A(3)2, which harbors a *vanHAX* gene cluster for the production of precursors ending in D-Lac, and *Nonomuraea* sp. strain ATCC 39727, which is devoid of *vanHAX* and produces the glycopeptide A40296. Vancomycin retained residual activity against *S. coelicolor* A(3)2 despite efficient incorporation of D-Lac into cytoplasmic precursors. This was due to a D,D-transpeptidase-catalyzed reaction that generated a stem pentapeptide recognized by glycopeptides by the exchange of D-Lac for D-Ala and Gly. The contribution of L,D-transpeptidases to resistance was limited by the supply of tetrapeptide acyl donors, which are essential for the formation of peptidoglycan cross-links by these enzymes. In the absence of a cytoplasmic metallo-D,D-carboxypeptidase, the tetrapeptide substrate was generated by hydrolysis of the C-terminal D-Lac residue of the stem pentadepsipeptide in the periplasm in competition with the exchange reaction catalyzed by D,D-transpeptidases. In *Nonomuraea* sp. strain ATCC 39727, the contribution of L,D-transpeptidases to glycopeptide resistance was limited by the incomplete conversion of pentapeptides into tetrapeptides despite the production of a cytoplasmic metallo-D,D-carboxypeptidase. Since the level of drug production exceeds the level of resistance, we propose that L,D-transpeptidases merely act as a tolerance mechanism in this bacterium.

Glycopeptide antibiotics, vancomycin and teicoplanin, bind to the peptidyl-D-alanyl-D-alanine termini of peptidoglycan precursors at the outer surface of the bacterial membrane and block peptidoglycan polymerization by steric hindrance (1). Resistance to glycopeptides by target modification is due to two mechanisms (2).

The first mechanism was discovered in clinical isolates of *Enterococcus faecium* that have acquired transposon Tn1546 (3) and resistance to high levels of vancomycin and teicoplanin by the production of peptidoglycan precursors ending in D-lactate (D-Lac) instead of D-Ala (4). Replacement of the NH group of the D-Ala-D-Ala amide bond with the oxygen of the D-Ala-D-Lac ester bond results in the loss of one of the five hydrogen interactions required for high-affinity binding of glycopeptides to the peptidoglycan precursors (5, 6). This substitution leads to a 1,000-fold reduction in the affinity of vancomycin for the target (5) and is sufficient for high-level resistance (MICs of >1,000 µg/ml), provided that the replacement of D-Ala with D-Lac is complete (7). This is achieved by hydrolysis of D-Ala-D-Ala by a dipeptidase (VanX) to enable efficient incorporation of the depsipeptide D-Ala-D-Lac (8), which is synthesized by the D-lactate dehydrogenase VanH (5, 9) and the D-Ala:D-Lac ligase VanA (5, 10, 11). The production of an accessory protein, the D,D-carboxypeptidase VanY, acts as a backup mechanism by cleavage of the C-terminal D-Ala residue of late peptidoglycan precursors inserted into the inner leaflet of the membrane (12). These precursors ending in D-Ala originate from incomplete VanX-mediated elimination of the dipeptide D-Ala-D-Ala produced by the host D-Ala:D-Ala li-

gase Ddl (12). Close homologues of VanH, VanA, and VanX (54 to 64% identity) have been detected in glycopeptide-resistant members of the order *Actinomycetales* that produce glycopeptides or share the same antibiotic-containing ecosystems, such as *Streptomyces coelicolor* (13–16).

The second glycopeptide resistance mechanism has been detected in mutants of *E. faecium* selected *in vitro* that are resistant to high levels of glycopeptides (MICs of >1,000 µg/ml) by the production of the metallo-D,D-carboxypeptidase DdcY in the absence of the production of precursors ending in D-Lac (17, 18). This enzyme eliminates the target of glycopeptides from peptidoglycan precursors by hydrolysis of the C-terminal D-Ala residue of pentapeptide stems. This modification accounts for resistance since the resulting tetrapeptide stems do not bind glycopeptides (6). However, this modification of the precursor, if complete, is usually lethal since the classical D,D-transpeptidases belonging to the penicillin-binding protein (PBP) family use precursors containing a pentapeptide stem as acyl donors (19). Cross-linking of tetrapeptide stems requires a PBP surrogate, an L,D-transpeptidase

Received 25 October 2013 Returned for modification 8 December 2013
Accepted 29 December 2013

Published ahead of print 6 January 2014

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doi:10.1128/AAC.02329-13

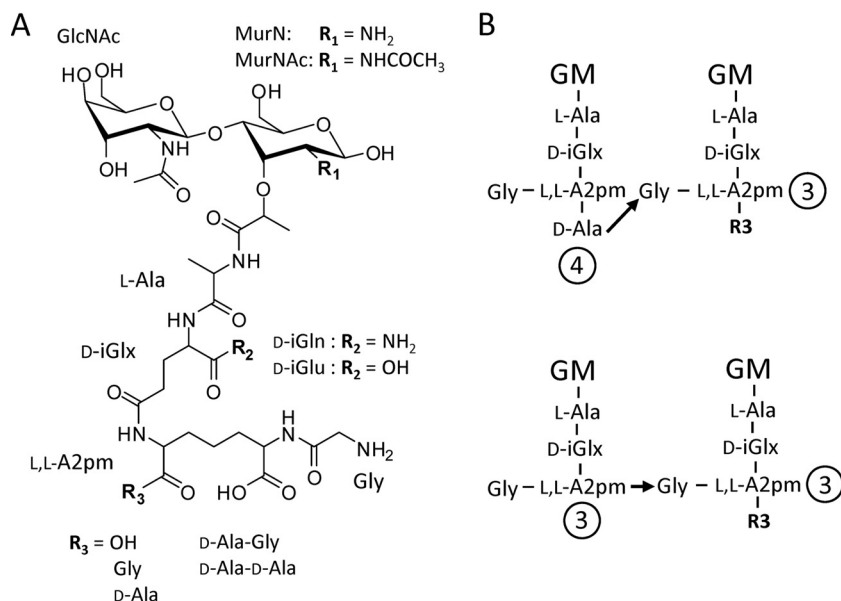


FIG 1 Structures of mucopeptides from *S. coelicolor* A(3)2. Polymorphisms are indicated for monomers (A) and dimers (B) generated by D,D-transpeptidases (4→3 cross-links) and L,D-transpeptidases (3→3 cross-links). D-iGlx, D-*iso*-glutamine or D-*iso*-glutamic acid; GM, GlcNAc-MurNAc or GlcNAc-MurN.

(LDT) (20–22). PBPs and LDTs are structurally unrelated, harbor different catalytic nucleophiles (Ser versus Cys), and catalyze the formation of different cross-links (4→3 versus 3→3), which connect the fourth or third residue of the acyl donor to the third position of the acyl acceptor, respectively (22, 23) (Fig. 1).

In this study, we investigated the peptidoglycan structures of *S. coelicolor* A(3)2 and *Nonomuraea* sp. strain ATCC 39727 to evaluate the contributions of the two mechanisms to the expression of glycopeptide resistance. *S. coelicolor* strain A(3)2 harbors a bona fide *vanHAX* gene cluster but does not produce any glycopeptide (15, 24). *Nonomuraea* sp. strain ATCC 39727 produces the glycopeptide A40296 and a DdcY-related metallo-D,D-carboxypeptidase but does not harbor any *vanHAX*-related gene cluster.

MATERIALS AND METHODS

Growth conditions. *S. coelicolor* A(3)2 and *Nonomuraea* sp. strain ATCC 39727 were grown in new minimal medium with phosphate (NMMP) broth or agar at 30°C.

Peptidoglycan extraction. *S. coelicolor* A(3)2 was grown to mid-exponential phase (24 h) in 500 ml of NMMP broth containing glass beads with vigorous shaking. Vancomycin (10 µg/ml) and glycine (20 mM) were added throughout growth as specified in Results. Bacteria were collected and disrupted with glass beads for 30 s (FastPrep), and peptidoglycan was extracted by the hot sodium dodecyl sulfate procedure as previously described (25). *Nonomuraea* sp. strain ATCC 39727 was grown for 48 and 108 h to analyze its peptidoglycan in the exponential and stationary phases of growth.

To prepare mucopeptides, peptidoglycan was serially treated with pronase, trypsin, mutanolysin, and lysozyme as previously described (25). Sodium borohydride and ammonium hydroxide were used to prepare reduced disaccharide and lactoyl peptides, respectively (25). Peptidoglycan fragments were separated by reversed-phase high-pressure liquid chromatography (rpHPLC) on a C_{18} column and detected by UV absorption at 210 nm, and their relative abundances were estimated as percentages of the total integrated area (25). The peaks were individually collected, lyophilized, and analyzed by electrospray time-of-flight mass spectrometry in the positive mode (Qstar Pulsar I; Applied Biosystems)

(25). Fragmentation was performed with nitrogen as the collision gas (25). The methods used for analysis of the spectra obtained by tandem mass spectrometry have been previously published (25, 26).

Extraction of peptidoglycan precursors. *S. coelicolor* A(3)2 was grown under the same conditions as those used for peptidoglycan extraction, except that precursor accumulation was obtained by incubation for an additional 1 h in the presence of bacitracin (100 µg/ml) (21). Peptidoglycan precursors were extracted with formic acid and purified by rpHPLC (21). Determination of the relative abundances and structures of the peptidoglycan precursors was performed as described above for peptidoglycan fragments, except that the absorbance at 280 nm was monitored.

RESULTS

Structure of mucopeptide monomers from *S. coelicolor* A(3)2.

The chemical structures of peptidoglycans from several bacteria belonging to the genus *Streptomyces* were determined by chemical approaches more than 40 years ago (27). We report here a high-resolution structure based on mass spectrometry. The structures of disaccharide peptides (mucopeptides) (Fig. 1A) were inferred from their molecular masses (Table 1) and fragmentation patterns obtained by tandem mass spectrometry (Fig. 2A to C; data not shown). Polymorphisms in mucopeptide monomers from *S. coelicolor* A(3)2 had three origins (Table 1 and Fig. 1A). First, N-acetylmuramic acid (MurNAc) was partially N-deacetylated (MurN). Second, the stem peptide consisted of a dipeptide, a tripeptide, a tetrapeptide, and a pentapeptide. Third, the α-carboxyl of D-*iso*-glutamic acid (D-iGlu) at the second position of stem peptides was partially amidated. In contrast, the ε-amino group of L,L-diaminopimelic acid (L,L-A2pm) at the third position of peptide stems was fully replaced with Gly. Efficient addition of Gly is due to two aminoacyl-tRNA transferases belonging to the Fem family, FemX and VanK, which are specific for the transfer of Gly from Gly-tRNA^{Gly} to peptidoglycan precursors under inducing and noninducing conditions, respectively (28).

Peptidoglycan cross-linking in *S. coelicolor* A(3)2. Polymor-

TABLE 1 Structures of peptidoglycan monomers of *S. coelicolor* A3(2)

Muropeptide ^a	Monoisotopic mass (Da)	
	Observed	Calculated
GlcNAc–MurNAc–L-Ala–D-Gln	697.30	697.30
GlcNAc–MurNAc–L-Ala–D-Glu	698.29	698.29
GlcNAc–MurN–L-Ala–D-iGln–L,L-A2pm(Gly)	884.40	884.40
GlcNAc–MurNAc–L-Ala–D-iGln–L,L-A2pm(Gly)	926.40	926.41
GlcNAc–MurN–L-Ala–D-iGlu–L,L-A2pm(Gly)	885.38	885.38
GlcNAc–MurNAc–L-Ala–D-iGlu–L,L-A2pm(Gly)	927.39	927.39
GlcNAc–MurN–L-Ala–D-iGln–L,L-A2pm(Gly)-D-Ala	955.44	955.43
GlcNAc–MurNAc–L-Ala–D-iGln–L,L-A2pm(Gly)-D-Ala	997.44	997.45
GlcNAc–MurNAc–L-Ala–D-iGln–L,L-A2pm(Gly)-D-Ala–D-Ala	1,068.49	1,068.48
GlcNAc–MurNAc–L-Ala–D-iGlu–L,L-A2pm(Gly)-D-Ala	998.43	998.43
GlcNAc–MurNAc–L-Ala–D-iGlu–L,L-A2pm(Gly)-D-Ala–D-Ala	1,069.47	1,069.47

^a D-iGln, D-*iso*-glutamine; L,L-A2pm(Gly), glycine linked to the side chain amine of L,L-A2pm.

phism in muropeptide dimers was generated by the coexistence of cross-links generated by D,D-transpeptidases (4→3) and L,D-transpeptidases (3→3), in addition to the variability detected in peptide stems described above (Fig. 1B). The two modes of cross-linking generated isomers that cannot be identified by mass spectrometry alone. For example, dimers with a monoisotopic mass of 1,089.49 Da may contain 4→3 or 3→3 cross-links, which differ by the presence of D-Ala at the fourth position of the acyl donor or acceptor stem, respectively (structures are shown in Fig. 2D). In order to sequence the cross-links, the disaccharide moiety of muropeptides was cleaved off and the resulting lactoyl peptides were analyzed by tandem mass spectrometry (Fig. 2D; data not shown). We sequenced the cross-links of all of the muropeptides listed in Table 2 by this approach and found that 57% of the cross-links were of the 3→3 type. These cross-links were generated by L,D-transpeptidases that cleave the L,L-A2pm³–D-Ala⁴ bond of the tetrapeptide stem of the acyl donor and link the carbonyl of L,L-A2pm³ to the side chain Gly of the acyl acceptor (L,L-A2pm³→Gly–L,L-A2pm³ cross-links) (Table 2). Cross-links of the 4→3 type (43%) were generated by D,D-transpeptidases (PBP) that cleave the D-Ala⁴–D-Ala⁵, D-Ala⁴–Gly, or D-Ala⁴–D-Lac⁵ bond of the donor and form D-Ala⁴→Gly–L,L-A2pm³ cross-links. Analysis of muropeptide trimers and tetramers (data not shown) revealed similar proportions of 3→3 and 4→3 cross-links. Overall, LDTs and PBPs made similar contributions to peptidoglycan cross-linking. Among members of the order *Actinomycetales*, the proportion of 3→3 cross-links is higher in mycobacteria (60 to 80%) (29–31) but lower in *Corynebacterium jeikeium* (38%) (32). The proportion of 3→3 cross-links is very low in bacteria that are not members of the order *Actinomycetales* (<10%) (2), except in *Clostridium difficile* (73%) (33).

Impact of *van* gene induction on the structure of monomers from *S. coelicolor* A3(2). The *vanHAX* gene cluster of *S. coelicolor* A3(2) is regulated by a two-component regulatory system (VanRS) and is inducible by vancomycin (15). The analysis of lactoyl peptides was repeated for *S. coelicolor* A3(2) grown in the presence of 10 μg/ml of vancomycin, revealing three stem peptides in monomers (Table 2). In comparison to uninduced conditions, exposure to vancomycin led to full amidation of D-iGlu. Inducible amidation of D-iGlu has been reported in *C. difficile* (34) but not in glycopeptide-resistant enterococci (17, 26). Induction also led to an increase in the relative proportion of pentapeptides

ending in D-Ala (from 8 to 33%), the appearance of a new type of stem pentapeptide (ending in Gly; 30%), and the disappearance of tripeptides. The remaining monomers contained tetrapeptide stems (37%). The increase in the relative abundance of pentapeptides ending in D-Ala in the absence of precursors ending in D-Lac was unexpected since induction of the *vanHAX* gene cluster by vancomycin should have opposite effects due to the synthesis of D-Ala–L-Lac by VanH and VanA and the elimination of D-Ala–D-Ala by VanX. The presence of stems ending in Gly was also unexpected since VanA preferentially ligates D-2-hydroxy acids, such as D-Lac, to D-Ala (5).

Structure of cytoplasmic peptidoglycan precursors of *S. coelicolor* A3(2). To explore the origin of the unexpected composition of stem peptides produced under inducing conditions, cytoplasmic peptidoglycan precursors were extracted from *S. coelicolor* A3(2) grown in the presence of 10 μg/ml of vancomycin. The culture was incubated for 1 h in the presence of bacitracin to block the dephosphorylation of undecaprenyl-phosphate and thereby obtain accumulation of nucleotide precursors. Under these conditions, a UDP-MurNAc-pentapeptide ending in Gly was not detected. A UDP-MurNAc-pentapeptide ending in D-Lac was the main cytoplasmic precursor (93%). Small amounts (7%) of a UDP-MurNAc-pentapeptide ending in D-Ala were also detected. Thus, D-Ala–D-Lac was efficiently incorporated at the C terminus of peptidoglycan precursors upon induction of the *vanHAX* operon. This reflects the hydrolysis of D-Ala–D-Ala by VanX resulting in the preferential addition of D-Ala–D-Lac produced by VanH and VanA to a UDP-MurNAc-tripeptide (15). Since D-Ala–Gly was not incorporated into cytoplasmic precursors, pentapeptide stems ending in Gly found in peptidoglycan fragments (Table 2) resulted from exchange of the C-terminal D-Lac residue for Gly in the periplasm. This reaction is likely to be catalyzed by classical D,D-transpeptidases since acyl enzymes formed by PBPs have been shown to react with free amino acids (35–37). Exchange of D-Lac for D-Ala may also contribute to the large pool of monomers ending in D-Ala–D-Ala.

Impact of *van* gene induction on the structure of dimers of *S. coelicolor* A3(2). Induction of the *vanHAX* gene cluster by vancomycin led to a modest and probably insignificant increase in the proportion of 3→3 cross-links (from 57 to 64%) (Table 2). Polymorphism in muropeptide dimers resulted from acceptor stems containing tripeptides and tetrapeptides. Muropeptide trimers had similar cross-links and acyl acceptor stem compositions (data not shown). Overall, these results indicate that induction of the *vanHAX* gene cluster and production of precursors ending in D-Lac had little impact on the relative contributions of LDTs and PBPs to peptidoglycan cross-linking.

Impact of supplementation of the culture medium with Gly. Peptidoglycan structure analysis was repeated for *S. coelicolor* A3(2) grown in the presence of 10 μg/ml of vancomycin or 20 mM Gly (Table 2). Under inducing conditions, the addition of Gly led to an increase in the proportion of pentapeptide stems ending in Gly in dimers (from 0 to 8%) but not in monomers (30 versus 28%). Thus, the addition of Gly to the culture medium marginally stimulated the PBP-mediated exchange of D-Lac⁵ for Gly. In contrast, the exchange of D-Ala⁴ for Gly was stimulated since tetrapeptide stems ending in Gly increased both in monomers (from 0 to 21%) and in dimers (from 0 to 60%). Thus, a high concentration of Gly in the culture medium was required for the exchange of D-Ala⁴ for Gly, a reaction known to be catalyzed by L,D-transpep-

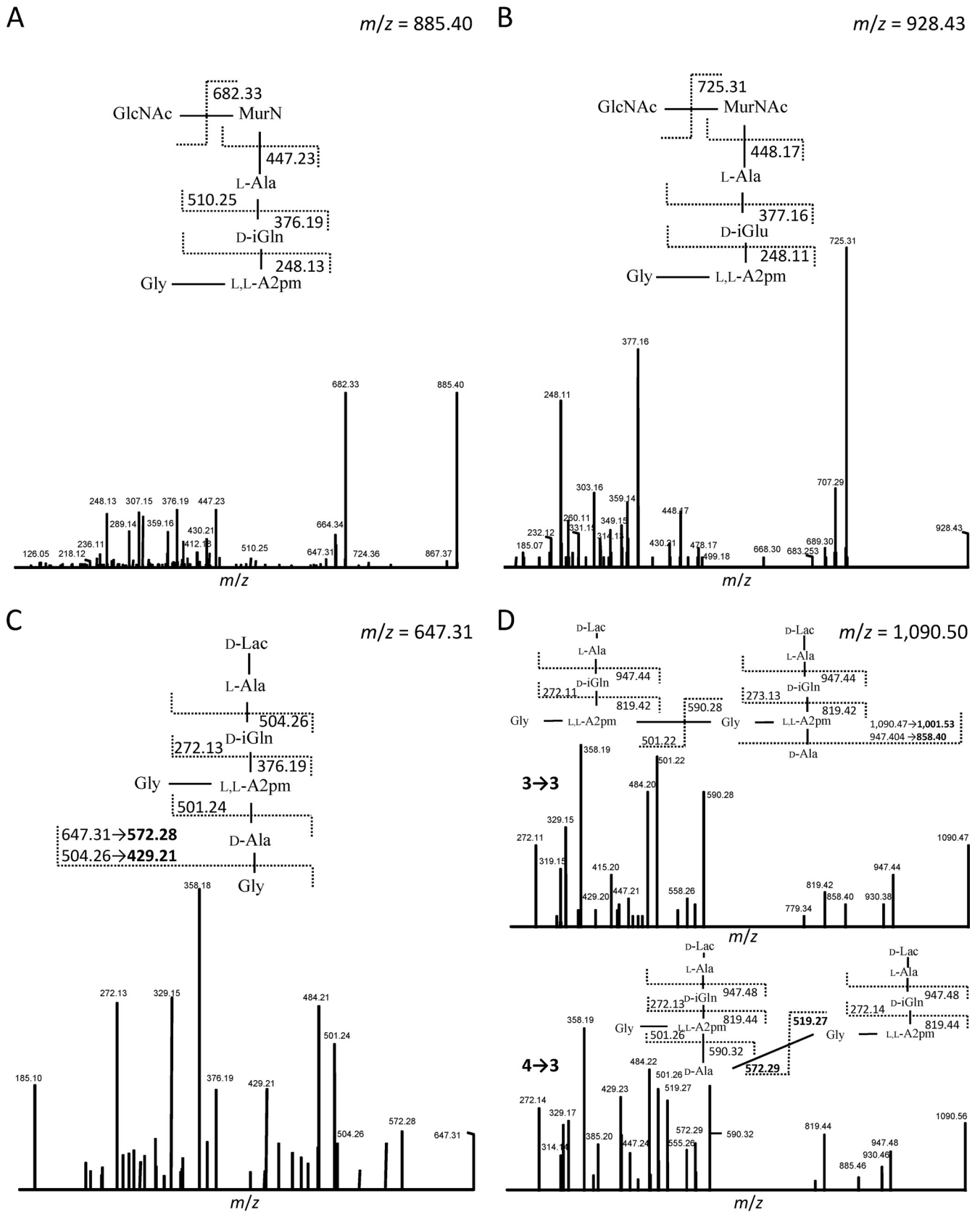


FIG 2 Tandem mass spectrometry analysis of peptidoglycan fragments. (A and B) Fragmentation of disaccharide peptides representative of polymorphisms resulting from N-deacetylation of MurNAc and amidation of D-iGlu, respectively. (C) Fragmentation of a lactoyl pentapeptide containing Gly both at the C terminus and in the side chain. (D) Sequencing of the cross-links of two isomers with 3→3 and 4→3 cross-links.

TABLE 2 Peptidoglycan fragments from *S. coelicolor* A3(2) grown under various conditions

Lactoyl peptide ^b or parameter	Calculated monoisotopic mass (Da)	Relative abundance (%) upon growth in NMMMP broth supplemented with ^c :		
		Nothing	Vm	Vm + Gly
Monomers				
Di	289.13	23	0	0
Tri	461.21	54	0	14
Tetra	532.25	16	37	17
Tetra (Gly) ^c	518.23	0	0	21
Penta	603.29	8	33	20
Penta (Gly) ^d	589.27	0	30	28
Dimers (cross-link)				
Tri-tri (3→3)	1,018.46	18	18	1
Tri-tetra (3→3)	1,089.49	39	46	8
Tri-tetra (Gly) ^c (3→3)	1,075.48	0	0	37
Tetra-tri (4→3)	1,089.49	22	0	0
Tetra-tetra (Gly) ^c (4→3)	1,146.52	0	0	23
Tetra-tetra (4→3)	1,160.53	13	36	23
Tetra-penta (4→3)	1,231.57	7	0	0
Tetra-penta (Gly) ^d (4→3)	1,217.55	0	0	8
Proportion (%) of 3→3 cross-links		57	64	46

^a The growth medium was supplemented with vancomycin (Vm) at 10 µg/ml or glycine (Gly) at 20 mM as indicated. The relative abundances of monomers and dimers were calculated independently.

^b The stem peptides linked to the D-lactoyl group consisted of dipeptides L-Ala-D-Glx (Di), tripeptides L-Ala-D-iGlx-L,L-A2pm(Gly) (Tri), tetrapeptides L-Ala-D-iGlx-L,L-A2pm(Gly)-D-Ala (Tetra), and pentapeptides L-Ala-D-iGlx-L,L-A2pm(Gly)-D-Ala-D-Ala (Penta). Lactoyl peptides containing D-iGlu and D-iGln were grouped (D-iGlx). Masses are provided for D-iGln-containing peptidoglycan fragments.

^c Gly at the fourth position of tetrapeptide stems in monomers and in the acceptor position of dimers.

^d Gly at the fifth position of pentapeptide stems in monomers and in the acceptor position of dimers.

tidas (22). The addition of Gly moderately decreased the proportion of dimers generated by L,D-transpeptidation (from 64 to 46%).

The analysis of cytoplasmic precursors was repeated for growth in the presence of vancomycin (10 µg/ml) or Gly (20 mM). Under these conditions, a UDP-MurNAc-pentadepsipeptide ending in D-Lac was the only precursor detected in the cytoplasm. The absence of a UDP-MurNAc-pentapeptide ending in Gly indicates that this amino acid was incorporated into muropeptides by the exchange reaction rather than by the ligation of glycine-containing substituents of D-Ala-D-Ala to a UDP-MurNAc-tripeptide in the cytoplasm. Likewise, the exchange reaction contributed to the formation of stem peptides ending in D-Ala⁴-D-Ala⁵ in mature peptidoglycan since a UDP-MurNAc-pentapeptide was not detected in the cytoplasm.

Peptidoglycan of *Nonomuraea* sp. strain ATCC 39727. Analysis of muropeptide monomers from exponentially growing bacteria revealed the presence of the disaccharide *N*-acetylglucosamine (GlcNAc)-MurNAc replaced with linear stem peptides containing D-iGlu and L,L-A2pm at the second and third positions (Table 3). Tetrapeptides were the most abundant monomers (85%), with D-Ala and Gly in similar proportions at the fourth position. The remaining monomers (15%) contained pentapep-

TABLE 3 Muropeptides from *Nonomuraea* sp. strain ATCC 39727 in the exponential and stationary phases of growth

Muropeptide or parameter	Calculated mass	Relative abundance (%) ^a	
		Exponential	Stationary
Disaccharide-peptide monomers^b			
Tetra	940.42	45	47
Tetra (Gly) ^c	926.41	40	43
Penta (Gly) ^d	997.45	15	10
Lactoyl-peptide dimers			
Tri-Tetra (Gly) (3→3) ^c	961.44	25	13
Tri-Tetra (3→3)	975.45	24	18
Tetra-Tetra (Gly) (4→3) ^c	1,032.47	28	7
Tetra-Tetra (4→3)	1,046.49	23	62
Proportion of 3→3 cross-links		49	31

^a Relative abundance was independently calculated for monomers and dimers.

^b The disaccharide consisted of MurNAc-GlcNAc. The stem peptide contained L-Ala, D-iGlu, and L,L-A2pm at the first, second, and third positions, respectively. Tri, tripeptide L-Ala-D-iGlx-L,L-A2pm; Tetra, tetrapeptide L-Ala-D-iGlx-L,L-A2pm-D-Ala; Penta, pentapeptide L-Ala-D-iGlx-L,L-A2pm-D-Ala-D-Ala.

^c Gly at the fourth position of tetrapeptide stems in monomers and in the acceptor position of dimers.

^d Gly at the fifth position of pentapeptide stems in monomers.

ptide stems with Gly at the fifth position. Pentapeptide stems ending in D-Ala or pentadepsipeptide stems ending in D-Lac were not detected. Monomers were similar in the exponential and stationary phases of growth.

Analysis of lactoyl dimers was performed to determine the relative proportions of 3→3 and 4→3 cross-links. During exponential growth, 49% of the cross-links were generated by L,D-transpeptidation. In the stationary phase, the proportion of 3→3 cross-links was lower (31%). The proportion of tetrapeptides containing Gly in the fourth position of the acceptor stem was also lower in dimers generated by L,D-transpeptidation (25 and 13%) and D,D-transpeptidation (28 and 7%) dimers. Thus, the contribution of L,D-transpeptidases to peptidoglycan metabolism appeared to be reduced during the stationary phase of growth with respect to both the formation of 3→3 cross-links and the exchange of D-Ala⁴ for Gly.

DISCUSSION

Analysis of peptidoglycan structure revealed that two mechanisms, production of precursors ending in D-Lac and L,D-transpeptidation, may contribute to glycopeptide resistance in *S. coelicolor* A3(2). First, a substantial majority of the cytoplasmic peptidoglycan precursors ended in D-Lac (93 to 100% in the absence or presence of Gly in the culture medium, respectively). Second, about half of the cross-links were generated by L,D-transpeptidases (Table 2), which use tetrapeptides as acyl donors. Neither pentadepsipeptides ending in D-Lac nor tetrapeptides bind glycopeptides with an affinity sufficient for inhibition of peptidoglycan polymerization (5, 6).

Despite the presence of these two mechanisms, vancomycin retained residual activity against *S. coelicolor* A(3)2 (MIC of 128 µg/ml versus 0.5 µg/ml for a Δ vanRS mutant) (38). We have previously shown that the levels of resistance to glycopeptides in the enterococci are determined by the extent of elimination of peptidoglycan precursors ending in D-Ala-D-Ala in both the Van-

HAX (7) and L,D -transpeptidase (17) resistance pathways. Binding of glycopeptides to small amounts of precursors containing the target of glycopeptides is sufficient to block the membrane translocation step of peptidoglycan synthesis due to sequestration of the undecaprenyl lipid carrier (7, 17). Quantitatively, a decrease in the proportion of precursors ending in D -Ala⁴- D -Ala⁵ from 100 to 50% leads to an only 2-fold increase in the MICs of glycopeptides (7, 18). Full elimination of precursors ending in D -Ala⁴- D -Ala⁵ (<2%) is required for high-level resistance (>500-fold increase in the MICs). In agreement with these analyses performed with enterococci, we show here that the levels of glycopeptide resistance are limited by the incomplete elimination of precursors ending D -Ala⁴- D -Ala⁵ in the members of the order *Actinomycetales*.

In *S. coelicolor* A(3)2, we have identified two factors that limit the levels of resistance mediated by the production of precursors ending in D -Lac. First, exchange of D -Lac for D -Ala contributes to the residual activity of vancomycin since this reaction generates the peptidyl- D -Ala- D -Ala target of glycopeptides in the periplasm. Exchange of D -Lac for Gly also generates pentapeptide stems recognized by the drugs since vancomycin was previously shown to bind model peptides ending in D -Ala- D -Ala and D -Ala-Gly (6). The affinity of vancomycin for the latter peptides is 10-fold lower because of the absence of a hydrophobic interaction involving the side chain methyl of the C-terminal D -Ala. Second, the chromosome of *S. coelicolor* A(3)2 does not contain any gene encoding a metallo- D,D -carboxypeptidase related to VanY, which contributes to glycopeptide resistance in the enterococci by eliminating precursors ending in D -Ala from the cytoplasm (12, 39). Heterospecific production of a VanY-related D,D -carboxypeptidase in *S. coelicolor* A(3)2 was previously reported to increase the level of resistance to the glycopeptide antibiotic balhimycin 5-fold (16). Thus, lack of production of a D,D -carboxypeptidase for hydrolysis of the C-terminal D -Ala of cytoplasmic precursors also limits the level of glycopeptide resistance in *S. coelicolor* A(3)2.

We have also identified several factors that limit the level of resistance conveyed by the second mechanism involving peptidoglycan cross-linking by L,D -transpeptidases in *S. coelicolor* A(3)2. In the absence of a metallo- D,D -carboxypeptidase for the formation of a UDP-MurNac-tetrapeptide in the cytoplasm, as found in highly resistant mutants of *E. faecium* (18, 20), the production of tetrapeptide stems in *S. coelicolor* A(3)2 depends upon hydrolysis of the C-terminal residue of precursors ending in D -Lac by low-molecular-mass PBPs in the periplasm. This reaction is likely to be efficient (40) but occurs in competition with the exchange of D -Lac for D -Ala by the D,D -transpeptidases in the periplasm. Further conversion of the resulting pentapeptide into tetrapeptide by low-molecular-weight PBPs is inhibited by glycopeptides. Thus, the exchange reaction is expected to prevent the formation of the essential substrate of the L,D -transpeptidases, thereby limiting the formation of 3→3 cross-links.

Nonomuraea sp. strain ATCC 39727 produces the glycopeptide A40296 and does not harbor any *vanHAX*-related gene cluster (41). This strain produces a metallo- D,D -peptidase related to VanY, which hydrolyzes the C-terminal D -Ala residue of stem pentapeptides, as well as D -Ala- D -Ala (42). *In vivo*, the enzyme acts in the cytoplasm and the ratio of UDP-MurNac-tetrapeptides to UDP-MurNac-pentapeptides rises from 3.2 to 9.2 upon induction by the glycopeptide A40296 (41). Gene inactivation in *Nonomuraea* sp. strain ATCC 39727 and heterospecific gene ex-

pression in *Streptomyces venezuelae* have shown that the D,D -carboxypeptidase confers a moderate level of resistance to glycopeptides (41, 42). Here, we show that a significant proportion of the cross-links were generated by L,D -transpeptidases in *Nonomuraea* sp. strain ATCC 39727 in both the exponential (49%) and stationary (31%) phases of growth (Table 3). Thus, the contribution of the D,D -carboxypeptidase to resistance is likely to result from the production of tetrapeptide stems that are cross-linked by L,D -transpeptidases in the presence of glycopeptides. However, incomplete elimination of D -Ala from peptidoglycan precursors accounts for the partial replacement of 4→3 cross-links with 3→3 cross-links and the moderate levels of resistance to vancomycin (MIC of 35 μ g/ml) and the glycopeptide A40296 (MIC of 5 μ g/ml) (41, 42).

The mechanism of self-resistance of *Nonomuraea* sp. strain ATCC 39727 is not efficient since the level of A40296 production in the culture medium (100 μ g/ml) by far exceeds the level of resistance to this glycopeptide (MIC of 5 μ g/ml) (41). However, the strain is fully tolerant to glycopeptides since no detectable killing occurs at glycopeptide concentrations as high as 5,000 μ g/ml (41). UDP-MurNac-tetrapeptide production by the metallo- D,D -carboxypeptidase and the subsequent use of tetrapeptide stems by L,D -transpeptidases therefore appear to be a mechanism of tolerance rather than a mechanism of resistance to glycopeptides. According to this hypothesis, tetrapeptide and L,D -transpeptidase production is essential when glycopeptides are removed from the external medium and bacteria resume growth. Under these circumstances, glycopeptides may remain bound to stems ending in D -Ala- D -Ala and the production of tetrapeptide-containing precursors may be essential for the reinitialization of peptidoglycan polymerization. It may appear paradoxical that *Nonomuraea* sp. strain ATCC 39727 produces a glycopeptide at a self-intoxicating concentration. This suggests that coproduction of the drug and precursors containing the drug target is a selective advantage for this bacterium. This could be the case if binding of the glycopeptide A40296 to a precursor ending in D -Ala- D -Ala has a protective role, for example, by blocking peptidoglycan hydrolysis by endogenous or exogenous hydrolases. In addition, glycopeptide production and expression of glycopeptide resistance genes may be used by the bacterium to reversibly downregulate peptidoglycan synthesis.

ACKNOWLEDGMENTS

We thank M.-J. Virolle for the generous gift of *S. coelicolor* A3(2).

This work was supported by National Institute of Allergy and Infectious Diseases grant RO1 AI046626. N.H. was funded by a research fellowship from the Région Ile-de-France. N.S. was funded by the European Community (EUR-INTAFAR, project LSHM-CT-2004-512138, sixth PCRD).

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