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Glycopeptides and β-lactams are the major antibiotics available for the treatment of infections due to Gram-positive bacteria. Emergence of cross-resistance to these drugs by a single mechanism has been considered as unlikely since they inhibit peptidoglycan polymerization by different mechanisms. The glycopeptides bind to the peptidyl-D-Ala[4]-D-Ala[5] extremity of peptidoglycan precursors and block by steric hindrance the essential glycosyltransferase and D,D-transpeptidase activities of the penicillin-binding proteins (PBPs). The β-lactams are structural analogues of D-Ala[4]-D-Ala[5] and act as suicide substrates of the D,D-transpeptidase module of the PBPs. Here we show that bypass of the PBPs by the recently described β-lactam-insensitive L,D-transpeptidase from *Enterococcus faecium* (Ldt_{fm}) can lead to high-level resistance to glycopeptides and β-lactams. Cross-resistance was selected by glycopeptides alone or serially by β-lactams and glycopeptides. In the corresponding mutants, UDP-MurNAc-pentapeptide was extensively converted to UDP-MurNAc-tetrapeptide following hydrolysis of D-Ala[5], thereby providing the substrate of Ldt_{fm}. Complete elimination of D-Ala[5], a residue essential for glycopeptide binding, was possible since Ldt_{fm} uses the energy of the L-Lys[3]-D-Ala[4] peptide bond for cross-link formation in contrast to PBPs which use the energy of the D-Ala[4]-D-Ala[5] bond. This novel mechanism of glycopeptide resistance was

unrelated to the previously identified replacement of D-Ala^5 by D-Ser or D-lactate.

Therapeutic usage of antibiotics has invariably led to emergence of resistance in target pathogens and members of commensal flora submitted to the same selective pressure. This scenario differs greatly between antibiotics with respect to the time period separating initial drug marketing and emergence of resistance (1). Detoxification of penicillin was recognized before the first therapeutic usage of the drug (2) and acquisition of resistance has immediately been a medical concern (3). In contrast, acquired resistance to glycopeptides was detected for the first time in enterococci 28 years after vancomycin had been launched in 1956 (4). During this unusually long delay, glycopeptides were considered as "irresistible" antibiotics and it has been rationalized that this class of drugs remained unharmed because of its unique mode of action (5). The drugs bind to the peptidyl-D-Ala^4-D-Ala^5 extremity of peptidoglycan precursors at the bacterial cell surface and block the transglycosylation and transpeptidation reactions by steric hindrance (Fig. 1A). This mode of action implies that acquisition of resistance cannot be easily achieved because modification of the target is limited by the specificity of multiple biosynthetic enzymes (5) including the Ddl ligase for synthesis of D-Ala-D-Ala, the MurF ligase for addition of the resulting dipeptide to the nascent cytoplasmic precursor, and the D,D-transpeptidases for peptidoglycan cross-linking in the last step of peptidoglycan polymerization (6). The latter reaction is catalyzed by penicillin-binding proteins (PBPs) that cleave the D-Ala^4-D-Ala^5 bond of a donor stem pentapeptide and link the carboxyl of D-Ala^4 to the amino group of the side chain carried by the third residue of an acceptor stem peptide (Fig. 1A).

Glycopeptide resistance has emerged in the enterococci (4) by acquisition of transposon Tn*1546* which mediates production of precursors terminating in D-lactate (D-Lac) instead of D-Ala (7, 8). The substitution leads to a 1,000-fold reduction in the affinity of vancomycin for its target (9). Tn*1546* encodes two biosynthetic enzymes for reduction of pyruvate into D-Lac (VanH) and synthesis of the depsipeptide D-Ala-D-Lac (VanA) (8). In addition, elimination of precursors ending in D-Ala is required for resistance. This function is carried out by two Tn*1546*-encoded D,D-peptidases which hydrolyzes D-Ala-D-Ala (VanX) and cytoplasmic precursors containing a stem pentapeptide (VanY) (10). Dissemination of Tn*1546* and related elements specifying the production of precursors ending in D-Ala-D-Lac is currently responsible for the high incidence of glycopeptide resistance in the enterococci and its emergence in methicillin-resistant *Staphylococcus aureus* (11, 12). In addition, low-level resistance to vancomycin can result from production of precursors ending in D-Ser (13, 14). Finally, substitution of D-Ala by D-2-hydroxybutyrate and D-2-hydroxyvalerate has been obtained in mutants constructed *in vitro* (15).

In this report, *in vitro* selection of high-level resistance to glycopeptides in *E. faecium* has been reevaluated in the light of our recent characterization of an L,D-transpeptidase (Ldt_{fm}) that specifically cross-links stem tetrapeptide lacking D-Ala^5 in a β-lactam insensitive reaction (Fig. 1B) (16). Since D-Ala^5 is essential for glycopeptide binding (17), peptidoglycan cross-linking by Ldt_{fm} cannot be inhibited by these drugs. Here, we show that activation of the L,D-transpeptidation pathway can also release inhibition of transglycosylation leading to cross-resistance to glycopeptides and β-lactams following extensive hydrolysis of D-Ala^5 from the cytoplasmic precursor UDP-MurNAc-pentapeptide by a D,D-carboxypeptidase. Characterization of this novel mechanism of glycopeptide resistance establishes for the first time that the fifth C-terminal residue of the stem peptide is dispensable for peptidoglycan polymerization.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Selection of Mutants Resistant to Glycopeptides – All cultures were performed at 37 °C in brain heart infusion (BHI) agar or broth (Difco Laboratories, Detroit, MI). A standard procedure was used for selection of mutants resistant to glycopeptides (18). Briefly, an inoculum of 4 X

10^9 colony forming units was prepared in broth and plated on agar containing 2-fold increasing concentrations of antibiotics. Selection steps that gave negative results were repeated with 1.3-fold increasing drug concentrations (selection of mutants G3 and G4). Mutants appeared after 3 to 5 days of incubation with a frequency of about 10^{-9} (1 to 15 colonies per plate). The selection procedure was repeated at each step with a mutant growing in the presence of the highest drug concentration. Spontaneous mutants G1 to G9 were obtained from *E. faecium* D344S (18) by using nine serial selection steps on agar containing increasing concentrations of glycopeptides. The concentrations of vancomycin were 4, 6, 8, 12, 16, 32, and 64 µg/ml for mutants G1, G2, G3, G4, G5, G6, and G7, respectively. Mutants G8 and G9 were selected with teicoplanin at 2 and 64 µg/ml, respectively. Mutants M6 to M9 were obtained from *E. faecium* M512 (18) by four serial selection steps on agar containing vancomycin at 16 (M6), 64 (M7), and 1,000 (M8) µg/ml or teicoplanin at 128 µg/ml (M9). Minimal inhibitory concentrations of ampicillin (Bristol-Myers, Paris, France), vancomycin (Merck, Lyon, France), and teicoplanin (Aventis, Paris, France) were determined by the agar dilution method after 48 h of incubation (18).

Preparation and Analysis of Cytoplasmic Peptidoglycan Precursors – Bacteria were grown to an optical density at 650 nm of 0.35 in 400 ml of BHI broth and treated with bacitracin (200 µg/ml) for 5 min. Ice-cold formic acid (47 ml, 1.1 M) was rapidly added to the bacterial pellet and nucleotide precursors were extracted for 30 min at 4°C. The extract was centrifuged (7,000 X *g* for 15 min at 4°C), the pH of the supernatant was adjusted to 6.0 with ammonium hydroxide, and lyophilized. Peptidoglycan precursors were analyzed by rp-HPLC with a C18 column (hypersil 4.6 by 250 mm, Interchim, Montluçon, France) at a flow rate of 0.5 ml/min with 50 mM ammonium formiate (pH 4.0). A methanol gradient (0-20%) was applied between 29 and 47 min and elution was continued for 20 min with 20% methanol. The relative abundance of the UDP-MurNAc-peptides was estimated by the percent of the integrate area of peaks detected by the absorbance at 262 nm. Products isolated by rp-HPLC were analyzed by mass spectrometry (MS) using an electrospray time-of-flight mass spectrometer operating in positive mode (Qstar Pulsar I, Applied Biosystem, Courtaboeuf, France) (19). Tandem mass spectrometry (MS-MS) was performed with nitrogen as the collision gas (19).

D,D-Carboxypeptidase Activity – Preparation of membrane extracts by sonication and determination of D-Ala release from L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala (Sigma-Aldrich, St. Louis, Mo) using D-amino oxydase coupled to peroxidase was performed as previously described (10). Ampicillin (20 µg/ml) was used to inhibit the D,D-carboxypeptidase activity of penicillin-binding proteins (18). D,D-carboxypeptidase activity was expressed as nmole of D-Ala produced per min and per mg of protein in the extract.

Peptidoglycan Structure Analysis – Bacteria were grown at 37°C to an optical density of 0.35 at 650 nm in 500 ml of BHI broth. Peptidoglycan was extracted with boiling SDS, treated with proteases, and digested with mutanolysin and lysozyme, as previously described (20). The resulting muropeptides were treated with ammonium hydroxide to cleave the ether link internal to MurNAc, separated by rp-HPLC, and analyzed by MS and MS/MS (20).

Western Blot Analysis – Anti Ldt_{fm} antiserum was obtained by three subcutaneous injections at 2 weeks intervals of 500 µg of purified protein (residues 119-466, ref. 16) in a New-Zealand rabbit. Bacteria were lysed in phosphate buffer (50 mM pH 7.0) with 0.17 µM glass beads (3 X 30s, FastPrep, Q-biogen, Illkirch, France). Proteins were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane (Hybond, Amerham, Biosciences, Little Chalfont, UK), and incubated with the anti Ldt_{fm} antiserum at a 1/1000 dilution. Goat anti rabbit IgG coupled to peroxidase (SouthernBiotech, Birmingham, AL) was used as secondary antibodies and Ldt_{fm} was detected by chemiluminescence (ECL kit, Pierce, Amersham Biosciences).

RESULTS

Sequential Acquisition of Ampicillin and Glycopeptide Resistance – We have previously

reported activation of the L,D-transpeptidation pathway (Fig. 1B) under the selective pressure of β-lactams (18, 21). Selection led to mutant M512 which is highly resistant to ampicillin whereas resistance to glycopeptides was not tested. As shown in Fig 2A, acquisition of high-level resistance to ampicillin in M512 (MIC >2,000 µg/ml) led only to a marginal increase (2-fold) in the MICs of vancomycin and teicoplanin. Thus, activation of the L,D-transpeptidation pathway was not sufficient in itself for glycopeptide resistance. Four additional steps of selection with glycopeptides were required to obtain mutant M9 that was cross-resistant to high levels of ampicillin and glycopeptides (Fig. 2A).

Acquisition of High-Level Cross-Resistance to Glycopeptides and Ampicillin under the Selective Pressure of Glycopeptides – E. faecium D344S was used to monitor the emergence of ampicillin-resistance under glycopeptide selection since this strain is highly susceptible to ampicillin due to the spontaneous deletion of the *pbp5* gene encoding a low-affinity penicillin-binding protein (PBP5) (18). Mutant G9 was obtained from D344S by nine serial selection steps on agar containing increasing concentrations of glycopeptides (Fig. 2B). The selection procedure led to 250- and 1,000-fold increases in the minimal inhibitory concentrations (MICs) of vancomycin (from 4 to 1,000 µg/ml) and teicoplanin (from 0.25 to 250 µg/ml). Even though ampicillin was not used, the selection procedure led also to the acquisition of high-level resistance to this antibiotic with a >36,000-fold increase in its MIC (from 0.06 to >2,000 µg/ml). This is the first report of emergence of cross-resistance to β-lactams under the selective pressure of glycopeptides.

UDP-MurNAc-Tetrapeptide is the Main Cytoplasmic Peptidoglycan Precursors of Mutant M9 and G9 – To gain insight into the mechanism of resistance, the structure of cytoplasmic peptidoglycan precursors was determined by mass spectrometry (Fig. 3 and Table 1). The parental strain D344S produced mainly UDP-MurNAc-pentapeptide (93 %) and small amounts of UDP-MurNAc-tetrapeptide (7%). Activation of the L,D-transpeptidation pathway in mutant M512 led to an increase in the pool of UDP-MurNAc-tetrapeptide from 7 to 60 %. The proportion of UDP-MurNAc-tetrapeptide was further increased in mutants M9 (>99 %) and G9 (92%). Thus, co-production of UDP-MurNAc-pentapeptide and UDP-MurNAc-tetrapeptide was associated with resistance to ampicillin only whereas extensive elimination of UDP-MurNAc-pentapeptide was associated with cross-resistance to ampicillin and glycopeptides. Strikingly, UDP-MurNAc-pentapeptide was undetectable in M9 indicating that the wild-type precursor is dispensable for peptidoglycan synthesis in this mutant.

Activation of a Cryptic D,D-Carboxypeptidase is Responsible for Accumulation of UDP-MurNAc-Tetrapeptide – Membrane extracts of the parental strain D344S contained low D,D-carboxypeptidase activity (Table 1). Inhibition by ampicillin indicated that hydrolysis of pentapeptide was mediated by the D,D-peptidase activity of PBPs (18). In contrast, mutants M512, M9, and G9 produced an additional D,D-carboxypeptidase that was insensitive to inhibition by ampicillin (Table 1). Thus, accumulation of UDP-MurNAc-tetrapeptide in the cytoplasm was due to hydrolysis of the UDP-MurNAc-pentapeptide by a D,D-carboxypeptidase that was not produced by the parental strain D344S. Surprisingly, the D,D-carboxypeptidase activity was not increased in mutant M9 in comparison to M512 in spite of an increase in the pool of UDP-MurNAc-tetrapeptide (from 60 to >99 %).

The Mutants M512, M9, and G9 Produce Wild-Type Enzymes for the Assembly of UDP-MurNAc-pentapeptide – The enzymes for incorporation of D-Ala at positions 4 and 5 of the nucleotide precursors (Fig. 1A) (6) were further investigated to eliminate the possibility that resistance to glycopeptides involved translocation of precursors carrying substitutions at the C-terminal positions of stem peptides. We also examined by the same approach the possibility that accumulation of UDP-MurNAc-tetrapeptide in the cytoplasm could result from production of modified precursors that would be more sensitive to hydrolysis than wild-type precursors.

DNA sequencing revealed that mutants M512, M9, and G9 harbor wild-type Ddl and MurF ligases for synthesis of the dipeptide D-Ala-D-Ala and its addition onto UDP-MurNAc-tripeptide, respectively (Fig. 1). Likewise, no

mutation was detected in the *asl*$_{fm}$ gene encoding the ligase for addition of the side chain D-aspartate residue to peptidoglycan precursors (22).

To confirm the *in vivo* specificity of these enzymes, the precursor pools of the mutants were thoroughly investigated to look for alternate precursors (data not shown). UDP-MurNAc-tripeptide was not detected indicating that the D-Ala-D-Ala substrate of the MurF ligase was not limiting, as previously found in glycopeptide-dependant enterococci (23). Precursors containing a side chain D-*iso*-aspartyl or D-*iso*-asparaginyl residue (D-iAsx) were present in both strains. Thus, the pathway for incorporation of D-Asp into the precursors and the subsequent amidation of its α-carboxyl group was not altered in the mutants.

Precursors containing D-Lac or D-Ser at the fifth position of stem peptides were not detected. Since the procedures used for the extraction of the precursors did not lead to the hydrolysis of D-alanyl-D-lactate ester bounds (data not shown), these results indicate that glycopeptide resistance in mutants M9 and G9 does not involve replacement of the C-terminal D-Ala residues of nucleotide precursors.

Total By-Pass of PBPs by Ldt$_{fm}$ *in Mutant M9* – The peptidoglycan of mutant M9 was analyzed by mass spectrometry to evaluate the contribution of the D,D-transpeptidase activity of PBPs and of the L,D-transpeptidase activity of Ldt$_{fm}$ to the formation of cross-links (Fig. 4). Sequencing of dimers by tandem mass-spectrometry (Fig. 5) indicated that the peptidoglycan manufactured by M9 during growth in the absence of antibiotic exclusively contained L-Lys^3→D-iAsx-L-Lys^3 cross-links generated by L,D-transpeptidation. This indicates that the donor substrate of the D,D-transpeptidases, which contains a stem pentapeptide, was not present in mutant M9 in agreement with the analysis of cytoplasmic peptidoglycan precursors (Table 1). In contrast, cross-links were both generated by L,D-transpeptidation and D,D-transpeptidation in mutant M512 grown in the absence of antibiotic (data not shown and ref. 18).

Analysis of the muropeptide composition was repeated for cultures of M9 performed in the presence of ampicillin (64 µg/ml), teicoplanin (32 µg/ml), or both antibiotics. The rp-HPLC profiles were very similar and mass spectrometry analysis did not reveal any modification of the structure of the lactoyl-peptides (data not shown). These observations confirm that glycopeptides and β-lactams had no effect on peptidoglycan synthesis in mutant M9.

A minority of the muropeptides of mutants M9 was found to contain Gly instead of D-Ala at the 4th position of stem peptides (Fig. 4). As glycine-containing precursors have not been detected in the cytoplasm, it is likely that incorporation of this residue results from exchange of D-Ala by Gly, a reaction previously shown to be catalyzed by Ldt$_{fm}$ *in vitro* (16). In conclusion, substitution of D-Ala^4→D-iAsx-L-Lys^3 by L-Lys^3→D-iAsx-L-Lys^3 cross-links and partial replacement of D-Ala^4 by Gly^4 were the only differences detected in the peptidoglycan of M9 in comparison to the peptidoglycan of wild-type strains of *E. faecium*.

Activation of the L,D-Transpeptidation Pathway in Response to The Selective Pressure of Glycopeptides in Mutant G9 – The muropeptide monomers from mutant G9 and the parental strain D344S were similar confirming that atypical precursors are not produced by mutant G9 (data not shown). The substantial majority (97 %) of the dimers from the susceptible strain D344S contained the usual D-Ala^4→D-iAsx-L-Lys^3 cross-links generated by the β-lactam-sensitive D,D-transpeptidase activity of the PBPs. The remaining dimers (3%) were produced by L,D-transpeptidation as they contained L-Lys^3→D-iAsx-L-Lys^3 cross-links. In contrast, the muropeptide oligomers of mutant G9 mainly contained these L-Lys^3→D-iAsx-L-Lys^3 cross-links. Tandem mass spectrometry indicated that modification of the cross-links and substitution of D-Ala^4 by Gly^4 were the only differences in the structure of the peptidoglycan from D344S and G9 (data not shown). Thus, the selective pressure of glycopeptides led to the activation of the L,D-transpeptidation pathway previously identified in ampicillin-resistant mutants of *E. faecium* (21). In contrast to M9, cross-links generated by D,D-transpeptidation were detected in a minority of the dimers of mutant G9. This difference correlates with the complete (< 1%) or partial (8 %) elimination of UDP-MurNAc-

pentapeptide from the cytoplasm of M9 and G9, respectively (Table 1).

DISCUSSION

β-lactams and glycopeptides are frequently the only alternative options for the treatment of severe infections due to Gram-positive bacteria. Acquired resistance to these antibiotics mainly results from two independent mechanisms involving production of low-affinity PBPs (24) and incorporation of D-Lac or D-Ser instead of D-Ala at the C-terminal position of peptidoglycan precursors (8). Neither of the two mechanisms confers alone cross-resistance and synergistic effects have been reported in certain strains of *E. faecium* in which inducible production of precursors ending in D-Lac increases susceptibility to β-lactams presumably because substitution of D-Ala by D-Lac impairs peptidoglycan cross-linking by low-affinity PBP5 (25). In this report, bypass of the PBPs by the Ldt_{fm} L,D-transpeptidase was investigated as a potential mechanism of cross-resistance, since the tetrapeptide substrate of Ldt_{fm} is not recognized by glycopeptides and since this enzyme is not inhibited by β-lactams. We show that cross-resistance can be selected by ampicillin and glycopeptides sequentially or by glycopeptides alone. The two selection schemes led to activation of the L,D-transpeptidation pathway which allowed extensive elimination of precursors containing the target of glycopeptides.

The first selection scheme revealed that high-level resistance to β-lactams and cross-resistance to glycopeptides can emerge sequentially under the selective pressure of ampicillin and glycopeptides. We have previously shown that selection by ampicillin leads to activation of the L,D-transpeptidation pathway (18, 21), but the resulting mutant, M512, remained susceptible to glycopeptides (Fig. 2A) and four additional selection steps with glycopeptides were required for cross-resistance (mutant M9). Hydrolysis of the C-terminal D-Ala^5 residue of cytoplasmic precursors was complete in M9 (<1% UDP-MurNAc-pentapeptide) whereas a significant proportion of the precursors of M512 contained the peptidyl-D-Ala^4-D-Ala^5 target of glycopeptides (40% UDP-MurNAc-pentapeptide). Thus, M512 produced sufficient amounts of UDP-MurNAc-tetrapeptide precursors to sustain peptidoglycan cross-linking by Ldt_{fm} leading to high-level resistance to ampicillin. However, sufficient amounts of UDP-MurNAc-pentapeptide remained for inhibition of transglycosylation by glycopeptides. Binding of the drugs to the pentapeptide stem of lipid intermediate II at the outer surface of the membrane is likely to account for inhibition of peptidoglycan polymerization since formation of this complex is expected to have two effects. First, the transfer of the disaccharide-pentapeptide subunit to growing glycan chains by the glycosyltransferases is blocked due to steric hindrance. Second, the lipid carrier is sequestered and cannot be recycled for incorporation of disaccharide-tetrapeptide units (Fig. 1). Similarly, production of D-Lac-ending precursors by the Tn*1546*-encoded VanH dehydrogenase and VanA ligase in sufficient amounts for peptidoglycan synthesis does not lead to glycopeptide resistance as complete elimination of pentapeptide stems prior to translocation of the precursors at the cell surface precursors is also required (26). This is mainly achieved by production of the VanX dipeptidase which cleaves the dipeptide D-Ala-D-Ala formed by the Ddl ligase and thereby limits synthesis of precursors ending in D-Ala^5 (10). In addition, a D,D-carboxypeptidases (VanY) hydrolyzes D-Ala^5 of late peptidoglycan precursors to completely eliminate precursors containing the target of glycopeptides (10). The resulting tetrapeptide stems cannot be used as donors by the D,D-transpeptidases (PBPs) since the energy for cross-link formation originates from the D-Ala^4-D-Ala^5 or D-Ala^4-D-Lac^5 bond (24). Thus, production of tetrapeptide by VanY releases inhibition of glycosyltransferases by glycopeptides although the resulting precursors cannot alone sustain peptidoglycan polymerization. In contrast, Ldt_{fm} uses the energy of L-Lys^3-D-Ala^4 bond and the fifth residue of peptide stems is therefore fully dispensable with this mode of peptidoglycan cross-linking.

The second selection scheme revealed that the selective pressure of glycopeptides alone can lead to cross-resistance to glycopeptides and β-

lactam antibiotics (Fig. 2B). The L,D-transpeptidation pathway was activated in the resulting mutant, G9, since its peptidoglycan contained mainly L-Lys^3→D-iAsx-L-Lys^3 cross-links. Production of precursors containing the tetrapeptide stem essential for the cross-linking activity of Ldt_{fm} was predominant in the cytoplasm of G9 (Fig. 3 and Table 1). An *in vitro* assay performed in the presence of ampicillin to inactivate the PBPs indicated that selection by glycopeptides led to activation of a cryptic D,D-carboxypeptidase which is not produced by the parental strain D344S (Table 1). This enzyme is therefore responsible for production of the tetrapeptide substrate of Ldt_{fm}, as previously described for mutant M512 (18). Sequencing of the *ldt_{fm}* gene and Western blot analysis using anti Ldt_{fm} antibodies (data not shown) did not reveal any difference between the parental strain D344S and the mutants M512, M9, and G9. Thus, activation of the L,D-transpeptidation pathway involved production of the tetrapeptide substrate of Ldt_{fm} rather than a modification of this enzyme. Since substitution at the 4th and 5th positions of stem peptides was excluded by detailed analyses of mature peptidoglycan and its precursors, these data establish that L,D-transpeptidation is a novel mechanism of glycopeptide resistance.

The structure of the dimers of mutant M9 (Fig. 4) indicated that two types of donor stems (tetrapeptide and tetrapeptide-iAsn) and two types of acceptor stems (tripeptide-iAsn and tetrapeptide-iAsn) were used for cross-link formation in all four possible combinations. Ldt_{fm} displays the same specificity *in vitro* (16). The enzyme is structurally unrelated to the PBPs, contains a catalytic Cys residue instead of Ser, and cross-links disaccharide-peptides in a β-lactam-insensitive L,D-transpeptidation reaction (16, 27). Thus, the specificity of Ldt_{fm} accounts both for the peptidoglycan structure of mutants M9 and G9 and the lack of inhibition of peptidoglycan cross-linking by ampicillin and glycopeptides since these drugs do not interact with the enzyme and its substrate, respectively. Considering the wide distribution of Ldt_{fm} homologues in eubacteria, this mechanism of cross-resistance may potentially emerge in various pathogens.

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FOOTNOTES

[1]The abbreviations used are: BHI, brain heart infusion; MIC, minimum inhibitory concentration; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PBP, penicillin-binding protein; rp-HPLC, reverse-phase high-pressure liquid chromatography.

FIGURE LEGENDS

Fig. 1. Peptidoglycan assembly in *Enterococcus faecium*. (*A*) In wild-type strains, β-lactams act as suicide substrates and inactivate the D,D-transpeptidase module of penicillin-binding proteins (PBPs). Glycopeptides bind to the peptidyl-D-Ala^4-D-Ala^5 extremity of peptidoglycan precursors and block transglycosylation by steric hindrance. Binding of the latter drugs to lipid intermediate II at the outer surface of the membrane (as represented) is thought to sequester the lipid carrier. Binding of glycopeptides to pentapeptide stems also inhibits the D,D-transpeptidase activity of the PBPs. (*B*) Activation of the L,D-transpeptidation pathway in mutants of *E. faecium* results from production of cytoplasmic precursors containing a tetrapeptide stem. The resulting precursors do not interact with glycopeptides and are cross-linked by the β-lactam-insensitive L,D-transpeptidase (Ldt_{fm}). D-iAsx; D-*iso*-aspartyl or D-*iso*-asparaginyl residue; D-iGln, D-*iso*-glutaminyl; D-iGlu, D-*iso*-glutamyl; GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid; P, phosphate..

Fig. 2. MICs of vancomycin, teicoplanin, and ampicillin for derivatives of *E. faecium* D344S. (*A*) Mutant M1, M2, M3, M4, and M512 were obtained by five consecutive selections steps on increasing concentration of ampicillin (18). Four additional selection steps with glycopeptides were required to obtain high-level cross-resistance to vancomycin, teicoplanin, and ampicillin. (*B*) Mutants G1 to G9 were obtained by serial selection with glycopeptides.

Fig. 3. MS/MS analysis of peak at *m/z* 1,079.35 corresponding to UDP-MurNAc-tetrapeptide. Cytoplasmic nucleotide precursors of mutant M9 were purified, separated by rp-HPLC, and identified by mass spectrometry and tandem mass spectrometry. Fragmentation of the peak at *m/z* 1,079.35 gave ions at *m/z* 675.27 and 490.19 corresponding to the MurNAc-tetrapeptide and lactoyl-tetrapeptide moieties of the molecule. Peak at *m/z* 401.21 matched the predicted value for loss of the D-alanine residue at the C-terminal position of lactoyl-tetrapeptide (*m/z* 490.19). Loss of additional L-Lys and D-Glu gave ion at *m/z* 273.14 and 144.05, respectively. The peaks at *m/z* 347.19, 258.12, 218.14, and 129.09 matched the expected mass of D-iGlu-L-Lys-D-Ala, D-iGlu-L-Lys, L-Lys-D-Ala, and L-Lys, respectively.

Fig. 4. Structure of the peptidoglycan of mutant M9. (*A*) rp-HPLC profile of lactoyl-peptides. Peptidoglycan was digested with muramidases and treated with ammonium hydroxide to cleave the ether link internal to MurNAc. This treatment also converts D-iso-asparaginyl into D-iso-aspartatyl (D-iAsp) residues. mAU, absorbance unit X 10^3 at 210 nm. (*B*) Schematic representation of dimers generated by L,D-transpeptidation. All multimers of M9 contained L-Lys^3→D-iAsp-L-Lys^3 cross-links. Variations occurred in the free side chain (presence or absence of D-iAsp) and at the 4th position of the acceptor stem peptide (presence of Gly, D-Ala or absence of a residue). The diversity of monomers resulted from variations at the same positions. (*C*) Peptidoglycan composition. The relative abundance (%) of the material in the peaks was calculated by integration of the absorbance at 210 nm. The structure of lactoyl-peptides was deduced from the mass and confirmed by tandem mass spectrometry for all monomers and dimers. Mass, observed monoisotopic mass.

Fig. 5. Sequencing of a dimer of M9 (peak 11) by tandem mass spectrometry. Fragmentation of the ion at *m/z* 1,118.5 (*A*) and inferred structure (*B*). Boxes indicate ions generated by cleavage at single peptide bounds. D-Lac, D-lactoyl.

Table 1. Characteristics of *E. faecium* D344S and derivatives selected on glycopeptide and β-lactam antibiotics.

	MICs (µg/ml) [a]			UDP-MurNAc-peptides (%) [b]		D,D-carboxypeptidase activity (nmol/min/mg) [c]	
Strain	**Vm**	**Te**	**Ap**	**Tetra**	**Penta**	**Without Ap**	**With Ap**
D344S	4	0.25	0.06	7	93	7.6 ± 3.1	< 1.0
M512	8	0.5	>2,000	60	40	38.3 ± 15.5	32.1 ± 11.6
M9	1,000	1,000	>2,000	> 99	< 1	18.5 ± 3.6	17.5 ± 5.5
G9	1,000	256	>2,000	92	8	50.7 ± 13.1	42.9 ± 10.7

[a] Vm, vancomycin ; Te, teicoplanin ; Ap, ampicillin.
[b] Bacteria were grown to an optical density of 0.35 at 650 nm and treated with bacitracin 200 µg/ml for 5 min to accumulate the precursors. After extraction with formic acid, nucleotide precursors were purified by rp-HPLC and identified by mass spectrometry. The relative abundance (%) of UDP-MurNAc-tetrapeptide (Tetra) and UDP-MurNAc-pentapeptide (Penta) was calculated by integration of absorbance at 262 nm.
[c] Hydrolysis of D-Ala from pentapeptide L-Ala-D-iGlu-L-Lys-D-Ala-D-Ala by D,D-carboxypeptidase activity in membrane fractions was determined using D-amino oxydase coupled to peroxidase in the presence (with Ap) or absence (without Ap) of ampicillin (20 µg/ml).

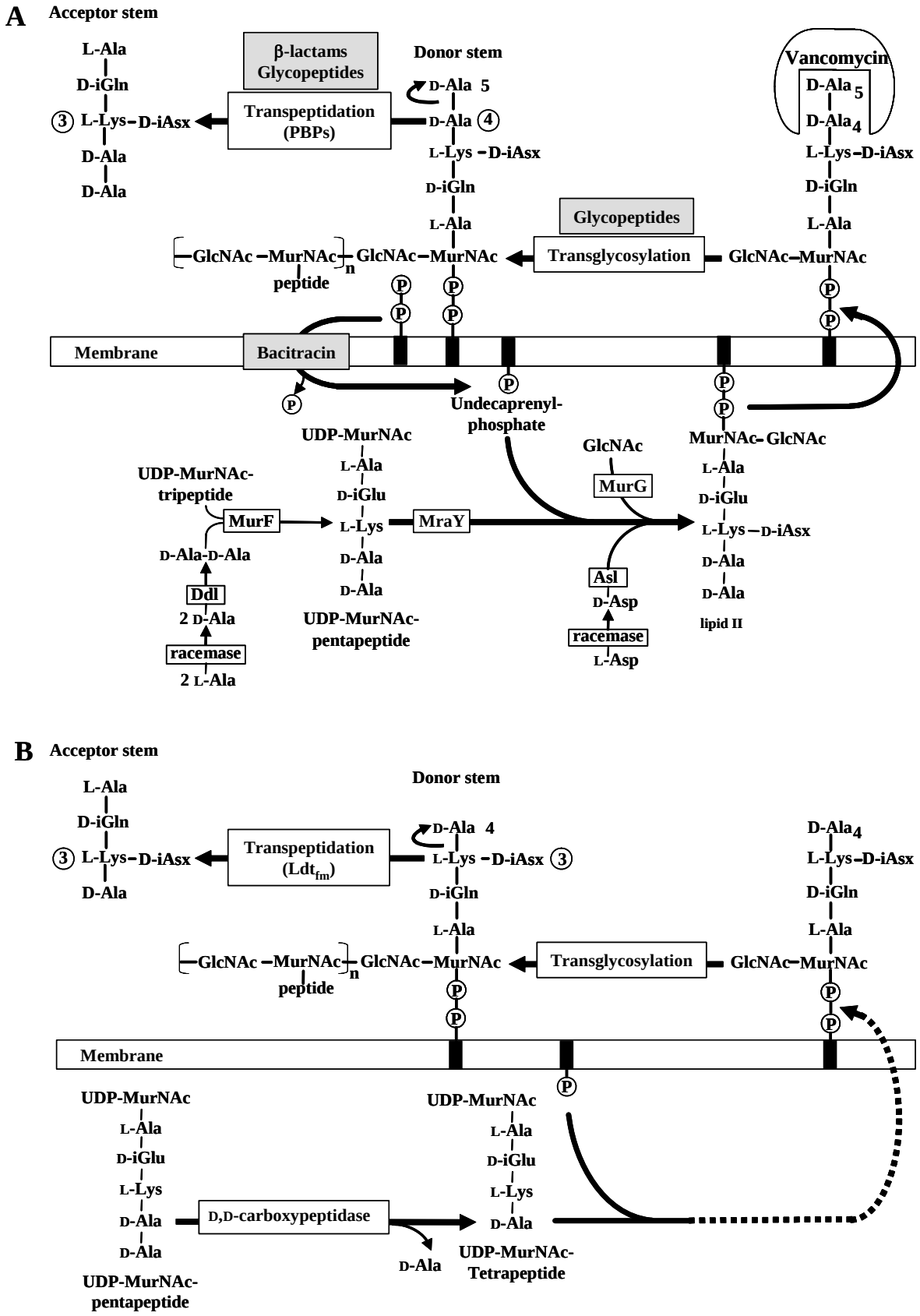


Fig. 1

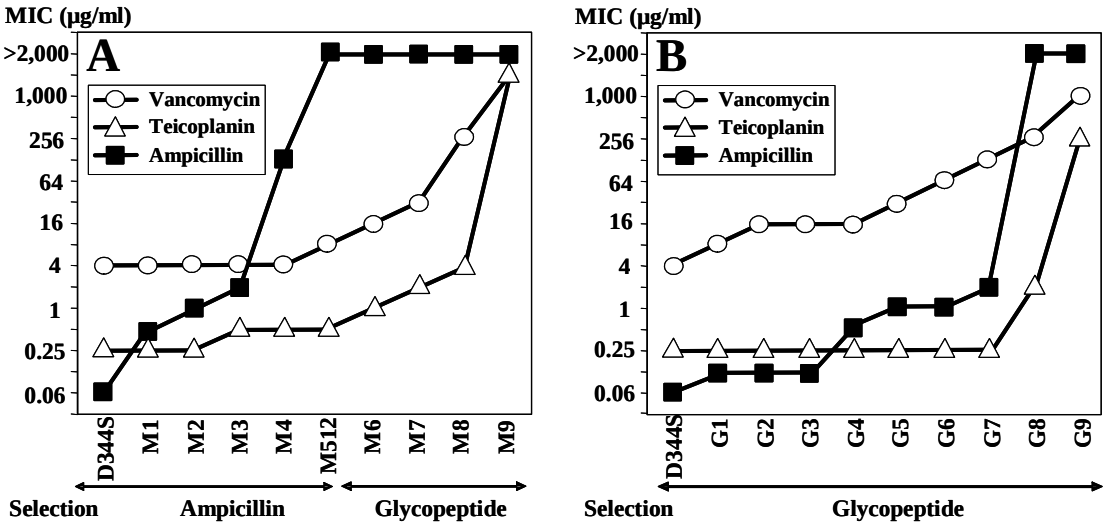


Fig. 2

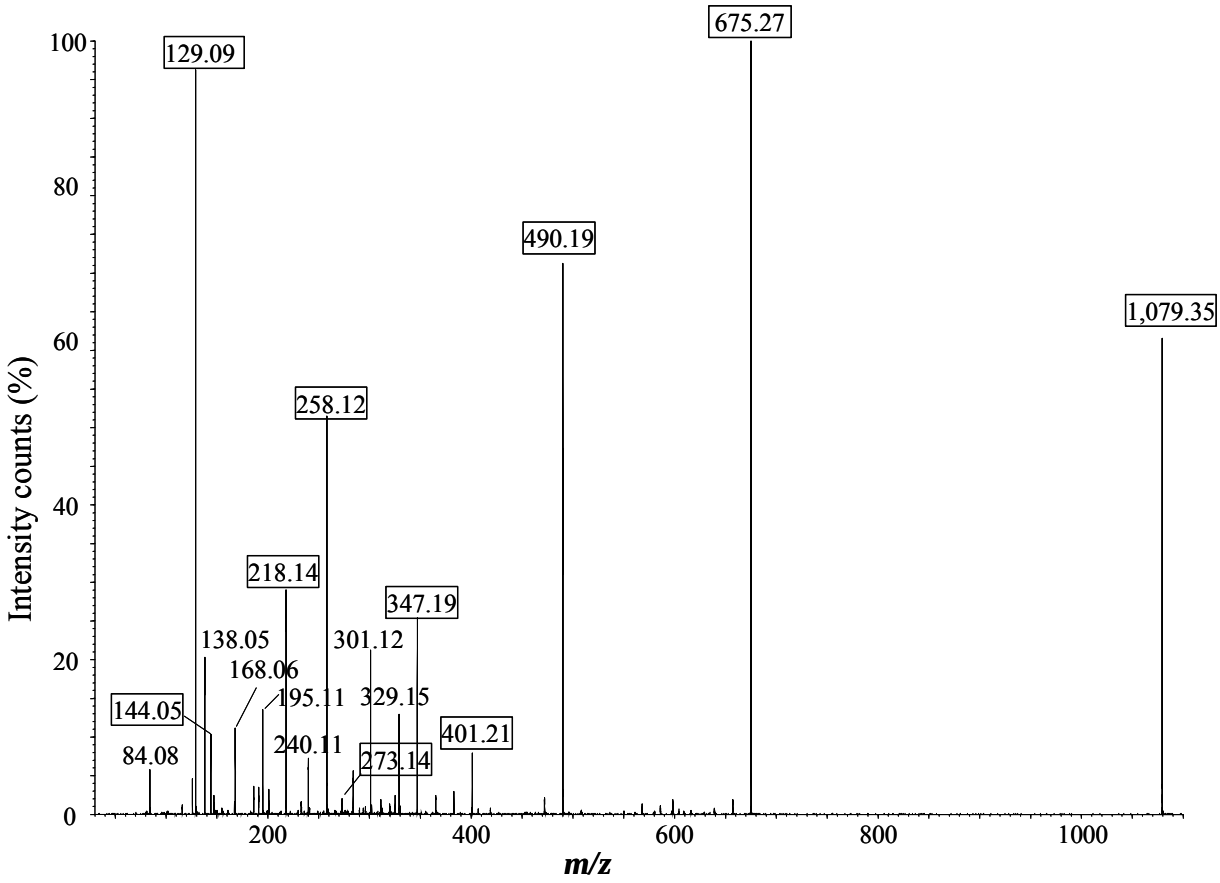
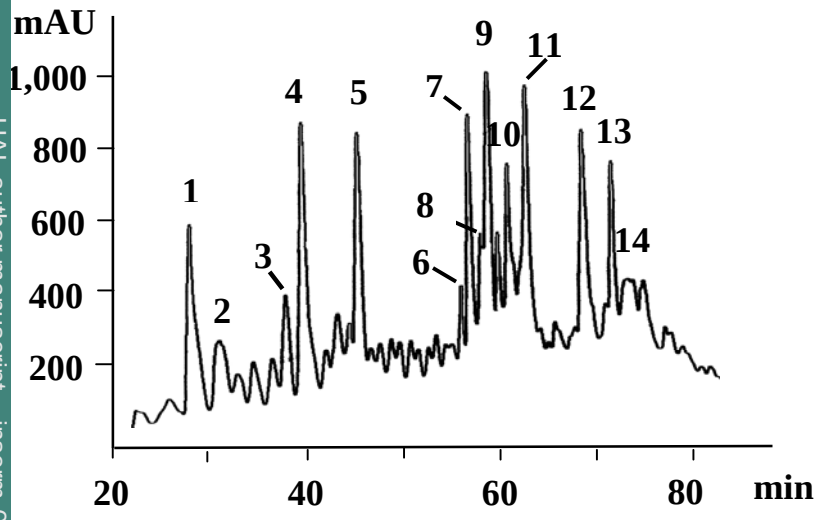


Fig. 3

A



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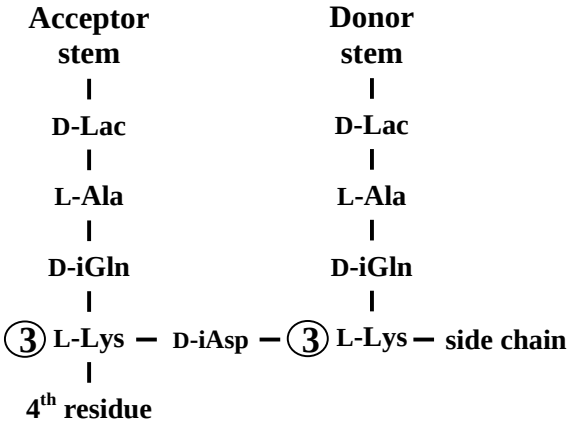
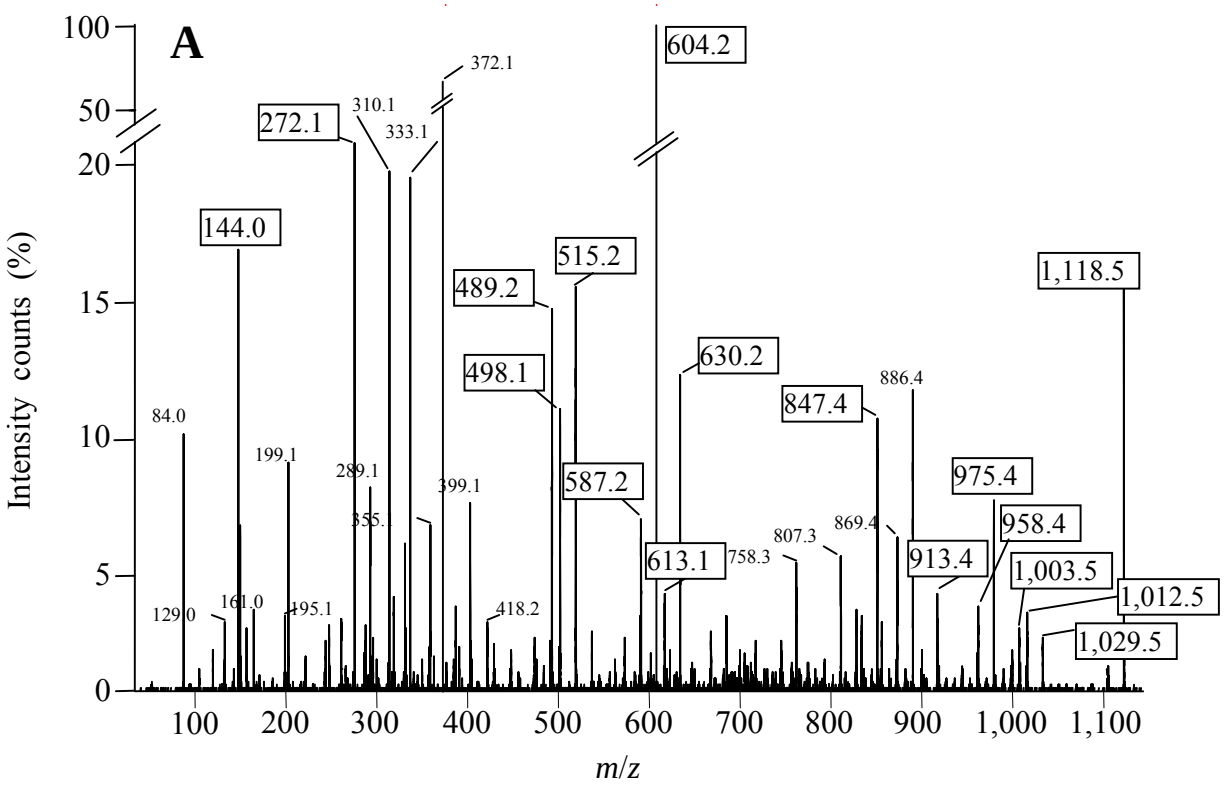


C

Peak	%	Mass	4th residue	Side chain
Monomers (43.0 %)				
1	11.0	417.22	None	None
2	5.5	474.25	Gly	None
3	4.4	488.25	D-Ala	None
4	12.2	532.22	None	D-Asp
		589.26	Gly	D-Asp
5	9.9	603.25	D-Ala	D-Asp
Dimers (40.7 %)				
6	1.4	988.42	Gly	None
7	7.5	931.39	None	None
8	3.3	1,103.41	Gly	D-Asp
9	11.5	1,046.44	None	D-Asp
10	6.0	1,002.41	D-Ala	None
11	11.0	1,117.40	D-Ala	D-Asp

Peak	%	Mass	4th residue	Side chain
Trimers (14.7 %)				
12	9.3	1,445.60	None	None
		1,560.60	None	D-Asp
13	5.3	1,516.70	D-Ala	None
		1,631.64	D-Ala	D-Asp
Tetramers (1.6%)				
14	1.6	2,016.90	Gly	None
		1,959.80	None	None
		2,074.89	None	D-Asp

Fig. 4



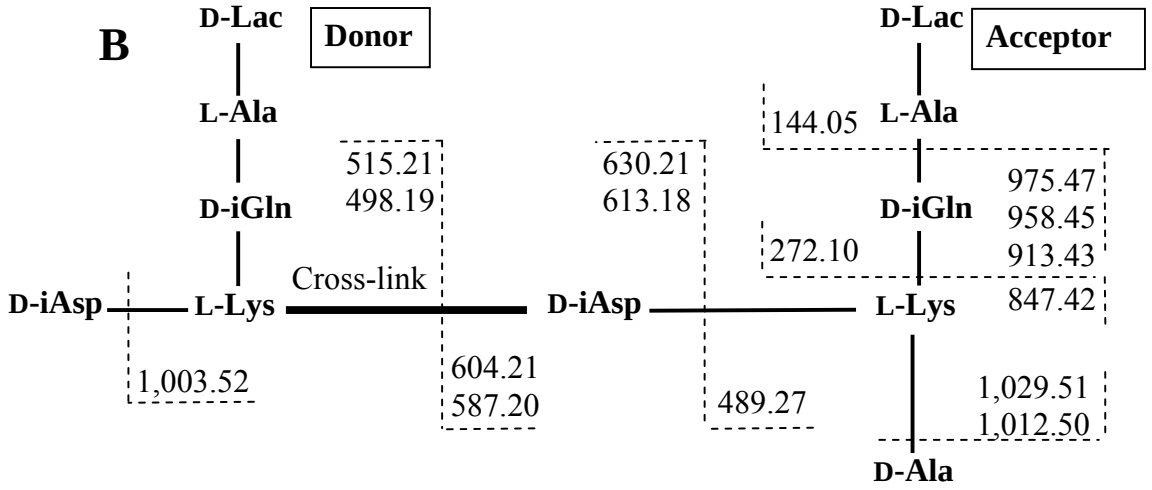


Fig. 5