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Specificity of L,D-transpeptidases from Gram-positive Bacteria Producing Different Peptidoglycan Chemotypes*

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We report here the first direct assessment of the specificity of a class of peptidoglycan cross-linking enzymes, the L,D-transpeptidases, for the highly diverse structure of peptidoglycan precursors of Gram-positive bacteria. The lone functionally characterized member of this new family of active site cystein peptidases, Ldt_{fm} from *Enterococcus faecium*, was previously shown to by-pass the D,D-transpeptidase activity of the classical penicillin-binding proteins

(PBPs) leading to high-level cross-resistance to glycopeptide and β-lactam antibiotics. Ldt_{fm} homologues from *Bacillus subtilis* (Ldt_{Bs}) and *E. faecalis* (Ldt_{fs}) were found here to cross-link their cognate disaccharide-peptide subunits containing *meso*-diaminopimelic acid (*meso*DAP³) and L-Lys³-L-Ala-Ala at the third position of the stem peptide, respectively, instead of L-Lys³-D-iAsn in *E. faecium*. Ldt_{fs} differed from Ldt_{fm} and

Ldt_{Bs} by its capacity to hydrolyze the L-Lys³-D-Ala⁴ bond of tetrapeptide (L,D-carboxypeptidase activity) and pentapeptide (L,D-endopeptidase activity) stems, in addition to the common cross-linking activity. The three enzymes were specific for their cognate acyl acceptors in the cross-linking reaction. In contrast to Ldt_{fs}, which was also specific for its cognate acyl donor, Ldt_{fm} tolerated substitution of L-Lys³-D-iAsn by L-Lys³-L-Ala-L-Ala. Likewise, Ldt_{Bs} tolerated substitution of *meso*DAP³ by L-Lys³-D-iAsn and L-Lys³-L-Ala-L-Ala in the acyl donor. Thus, diversification of the structure of peptidoglycan precursors associated with speciation has led to a parallel evolution of the substrate specificity of the L,D-transpeptidases affecting mainly the recognition of the acyl acceptor. Blocking the assembly of the side chain could therefore be used to combat antibiotic resistance involving L,D-transpeptidases.

The bacterial cell wall peptidoglycan is a net-like macromolecule which surrounds the cytoplasmic membrane (1). The polymer is essential since it supplies the cell with mechanical protection against the osmotic pressure of the cytoplasm. The peptidoglycan subunit contains β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) substituted by a peptide stem (Fig. 1A) (2). Assembly of the subunit at the cell surface is performed by glycosyltransferases that polymerize the glycan strands by formation of β-1,4 bonds and D,D-transpeptidases that cross-link glycan strands (2). The latter reaction is catalyzed by penicillin-binding proteins (PBPs) that cleave the D-Ala⁴-D-Ala⁵ bond of a donor stem pentapeptide and link the carbonyl of D-Ala⁴ to the amino group of the side chain carried by the third residue of an acceptor stem peptide (Fig. 1B). This two-step reaction involves formation of a covalent adduct between the β-hydroxyl of the active site serine of the PBPs and the carbonyl of D-Ala₄ of the donor stem (3). The glycosyltransferase and D,D-transpeptidase activities of the multimodular peptidoglycan polymerases are the targets of the two major classes of antibiotics available to treat severe infections due to Gram-positive bacteria, the β-lactams and the glycopeptides, that act by

different mechanisms. The β-lactams are structural analogues of D-Ala⁴-D-Ala⁵ and act as suicide substrates of the D,D-transpeptidase module of the PBPs. The glycopeptides bind to the peptidyl-D-Ala⁴-D-Ala⁵ extremity of peptidoglycan precursors and block by steric hindrance both the transglycosylation and transpeptidation reactions (4).

The assembly pathway of peptidoglycan precursors and its mode of polymerization are generally highly conserved in eubacteria. Variations in the structure of peptidoglycan subunits involve mainly the fifth (C-terminal) and third positions of the pentapeptide stem (5). The specificity of peptidoglycan cross-linking enzymes for the donor is the bottle neck that limits emergence of resistance to glycopeptides since the modifications of the precursors that prevent drug binding should be tolerated by these enzymes (4,6). Successful modifications which have spread in Gram-positive pathogens under the selective pressure of glycopeptides include incorporation of D-lactate or D-Ser instead of D-Ala at the 5th position of pentapeptide stems (7). Strikingly, production of D-lactate-ending precursors can also have an impact on the activity of β-lactams in the enterococci and staphylococci, presumably because low-affinity PBPs responsible for β-lactam resistance cannot function with modified precursors (6,8). Total elimination of D-Ala⁵ by hydrolysis of the C-terminal residue of pentapeptide stems is an alternative mechanism of glycopeptide resistance in mutants of *Enterococcus faecium* selected in laboratory conditions (9). Since PBPs cannot function with tetrapeptide donors, peptidoglycan cross-linking in these mutants requires an L,D-transpeptidase (Ldt_{fm}) that cleaves the L-Lys³-D-Ala⁴ peptide bond of the donor and links the carboxyl of L-Lys³ to the side chain amine of the acceptor (Fig. 1B). This mode of peptidoglycan cross-linking has been originally identified as a by-pass of the PBPs that confers high-level β-lactam resistance (10).

Variation at the third position of the peptide stem concerns both the nature of the diamino acid present at this position, e.g. L-Lys or *meso*-diaminopimelic acid (*meso*DAP), and the presence or absence of a side chain comprising from one to five amino acids (5) (Fig. 1A).

Glycine and L-amino acids are incorporated into the side chain of peptidoglycan precursors by transferases of the Fem family that use aminoacyl-tRNAs as the substrate (11). D-aspartic acid is activated as β -aspartyl-phosphate and ligated to the precursors by ATP-dependent ligases belonging to the ATP-Grasp superfamily (12). Synthesis of complete side chains is essential for β -lactam resistance mediated by low-affinity PBPs in Gram-positive bacteria, in particular methicillin resistance mediated by PBP2a in *Staphylococcus aureus* (13,14) and penicillin resistance mediated PBP2X in *Streptococcus pneumoniae* (15). For this reason, Fem transferases are considered as attractive targets for the development of novel antibiotics active against β -lactam-resistant pathogens (16,17). The antibacterial activity of such Fem inhibitors will ultimately depend upon the incapacity of cross-linking enzymes to use precursors with incomplete side chains.

Interaction of the peptidoglycan cross-linking enzymes with their substrates has not been extensively investigated, despite its pivotal role for drug development and for our understanding of the mechanisms of resistance to glycopeptides and β -lactams. In this report, the specificity of Ldt_{fm} and of the PBPs was compared based on heterospecific expression of a Fem transferase of *E. faecalis* in *E. faecium* and search for modified stem peptides containing L-Lys³-L-Ala instead of L-Lys³-D-iAsn in the donor and acceptor positions of dimers generated *in vivo* by L,D-transpeptidation and D,D-transpeptidation. The specificity of Ldt_{fm} was also studied *in vitro* by directly testing the cross-linking of peptidoglycan fragments isolated from three bacterial species (*E. faecium*, *Bacillus subtilis*, and *E. faecalis*) representative of the structural variability at the third position of the stem peptides (Fig. 1A). Finally, Ldt_{fm} homologues (Fig. 1C) were purified from *E. faecalis* and *B. subtilis* to compare the specificity of enzymes from bacteria producing peptidoglycan of different chemotypes. This analysis represents the first direct assessment of the specificity of peptidoglycan cross-linking enzymes since the PBPs are generally inactive *in vitro*. In addition, characterization of Ldt_{fs} revealed that members of the L,D-transpeptidase

family can also display L,D-carboxypeptidase and L,D-endopeptidase activities.

EXPERIMENTAL PROCEDURES

Production and Purification of L,D-transpeptidases—A catalytically active fragment of Ldt_{fm} from *E. faecium* M512 (residues 119 to 466) was produced in *E. coli* and purified by affinity, anion exchange, and size exclusion chromatographies, as previously described (18).

A fragment of the open reading frame encoding residues 136 to 474 of the L,D-transpeptidase of *E. faecalis* strain JH2-2 (19), Ldt_{fs}, was amplified with primers 5'-AACCATGGGGAGTATCCGTCGAGGCAATGG-3' and 5'-AAGGATCCTACTTCTTCGCCGTAATCTA-3'. The PCR product was digested with NcoI and BamHI (underlined) and cloned into pET2818, a derivative of pET2816 (18) lacking the sequence specifying the thrombin cleavage site. The resulting plasmid, pET2818 Ω Ldt_{fs}, was introduced in *E. coli* BL21(DE3) harboring pREP4GroESL (20) and bacteria were grown at 37°C to an optical density at 600 nm of 0.8 in brain heart infusion broth (Difco, Elancourt, France) containing ampicillin (100 μ g/ml). Isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 0.5 mM and incubation was continued for 17 hours at 16°C. The cells were disrupted by sonication in 50 mM Tris-HCl pH 7.5 containing 300 mM NaCl and cell debris were removed by centrifugation. Ldt_{fs} was purified from the resulting clarified lysate by affinity chromatography on Ni²⁺-nitrilotriacetate-agarose resin (Qiagen GmbH, Hilden, Germany). Proteins eluted with 200 mM imidazole were dialyzed against 50 mM Tris-HCl (pH 8.0) containing 60 mM NaCl, loaded onto an anion exchange column (MonoQ HR5/5, Amersham Biosciences, Saclay, France) equilibrated with the same buffer. Ldt_{fs}, eluting at approximately 300 mM NaCl, was further purified by size exclusion chromatography on a Superdex HR10/30 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl. The protein was obtained with an overall yield of 3 mg per liter of culture, as estimated by the BioRad Protein Assay using bovine serum albumin as a standard. The protein was stored at -80°C in 50

mM Tris-HCl (pH 7.5) containing 300 mM NaCl.

The open reading frame encoding the L,D-transpeptidase of *B. subtilis* strain 168, Ldt_{Bs}, previously named ykuD (21), was amplified with primers 5'-AACCATGGGGCTGCTTACGTACCAGGTG AAGC-3' and 5'-TTGGATCCCCGGTTAATCGTGACTCTCGT-3'. The PCR product digested with NcoI and BamHI (underlined) was cloned into pET2818 and Ldt_{Bs} was produced in *E. coli* BL21(DE3)/pREP4GroESL using the inducing conditions described above for the L,D-transpeptidase of *E. faecalis*. Ldt_{Bs} was purified in one step by affinity chromatography on Ni²⁺-nitrilotriacetate-agarose resin (Qiagen), dialyzed against 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, and stored at -20° C in the same buffer. Ten mg of protein were obtained per liter of culture.

L,D-transpeptidase assays—The source of the disaccharide-peptides used as substrates was as follows. The disaccharide-tetrapeptide substituted by a D-iso-asparagin residue (L-Lys³-D-iAsn) was purified from the peptidoglycan of *E. gallinarum* strain SC1 (22). The disaccharide-pentapeptide and the disaccharide-tetrapeptide substituted by an L-Ala-L-Ala side chain (L-Lys³-L-Ala-L-Ala) were purified from *E. faecalis* JH2-2 (19) and from a derivative of strain JH2-2 harboring the *vanA* glycopeptide resistance gene cluster (23), respectively. The disaccharide-tetrapeptide containing meso-diaminopimelic acid (*meso*DAP³) was purified from *E. coli* strain ATCC 25113. The procedures used for peptidoglycan preparation, digestion with muramidases, and reduction of MurNAc to muramitol with sodium borohydride have been previously described for enterococci (24) and *E. coli* (25). The resulting muropeptides were separated by rp-HPLC in acetonitrile gradients containing trifluoroacetic acid (24) and identified by mass spectrometry (MS). The concentration of the muropeptides was estimated by amino acid analysis after acidic hydrolysis with a Hitachi autoanalyser (26).

In vitro formation of muropeptide dimers was tested in 10 µL of phosphate buffer (20 mM, pH 7.0) containing the L,D-transpeptidase (7 µM) from *E. faecium* (Ldt_{fm}), *E. faecalis* (Ldt_{fs}),

or *B. subtilis* (Ldt_{Bs}) and a combination of three reduced disaccharide-tetrapeptides (200 µM each) containing L-Lys³-D-iAsn, L-Lys³-L-Ala-L-Ala or *meso*DAP³ at the third position of a tetrapeptide stem. The reaction was incubated for 2 hours at 37°C, desalted using a micro column (ZipTipC₁₈, Millipore, Saint Quentin-en-Yvelines, France), and analyzed by nanoelectrospray MS in the positive mode (Qstar Pulsar I, Applied Biosystem, Courtaboeuf, France). The sequence of the cross-links in dimers generated *in vitro* was determined by tandem mass spectrometry (MS/MS). Briefly, dimers were generated *in vitro* as described above except that the disaccharide-peptides used as substrates were not reduced. The reaction mixture was treated with ammonium hydroxide, desalted using a micro column (ZipTipC₁₈, Millipore), and the resulting lactoyl-peptides were analyzed by nanoelectrospray MS/MS using N₂ as the collision gas (24).

The L,D-transpeptidase activity of Ldt_{fs} was also tested by using the dipeptides L-Ala-L-Ala and D-Ala-D-Ala as acyl acceptors. The assay performed in 10 µL of 20 mM potassium phosphate buffer (pH 7.0) contained Ldt_{fs} (7 µM), the cognate disaccharide-tetrapeptide containing an L-Ala-L-Ala side chain as the acyl donor (200 µM), and 1 mM of L-Ala-L-Ala or D-Ala-D-Ala (Sigma). The products of the reactions were identified by MS and MS/MS, as previously described (18).

*Expression of the *bppA1* Gene of *E. faecalis* in *E. faecium* M512 and Analysis of Peptidoglycan Structure*—The *bppA1* gene of plasmid pDA15 (27) was subcloned under the control of the inducible promoter of pJEH4 (12) using XbaI and KpnI. The resulting plasmid, pJEH6(*bppA1*), was introduced by electroporation into *E. faecium* M512 (10). The recombinant strain was grown in brain heart infusion broth or agar (Difco) containing spectinomycin (120 µg/ml) to counter select loss of pJEH6(*bppA1*). Induction of the *bppA1* gene was performed with 0.3 mM isopropyl-β-D-thiogalactopyranoside at an optical density at 600 nm of 0.02. Incubation was continued at 37°C until the optical density reached 0.6 and bacteria were collected by centrifugation. Peptidoglycan was extracted with boiling SDS and digested with mutanolysin and lysozyme

(Sigma-Aldrich) (24). The resulting muropeptides were cleaved under alkaline conditions to generate lactoyl-peptides, separated by rp-HPLC, and analyzed by MS and MS/MS (24).

RESULTS AND DISCUSSION

Probing the Substrate Specificity of Ldt_{fm} in Vivo—Synthesis of the side chain of peptidoglycan precursors is catalyzed in *E. faecalis* by two members of the Fem family, BppA1 and BppA2, that sequentially add two L-Ala residues (Fig. 1A) (23). In *E. faecium*, D-Asp is added to the precursors by the Asl_{fm} ligase and subsequently partially amidated (12). The *bppA1* gene of *E. faecalis* was cloned under the control of an inducible promoter to generate plasmid pJEH6 and introduced into *E. faecium* M512 in order to manipulate the structure of the substrate of the cross-linking reaction which can be catalyzed in this mutant by Ldt_{fm} and by the PBPs (28). The BppA1 transferase efficiently competed with the Asl_{fm} ligase in *E. faecium* M512/pJEH6(*bppA1*) since the main monomers contained L-Ala instead of D-iAsp (peaks 1 and 2 in Fig. 2). The free side chains in the major dimers generated by L,D- and D,D-transpeptidation also contained L-Ala indicating that Ldt_{fm}, as the PBPs, had catalyzed cross-link formation using donors containing this residue (Peaks 3, 4, and 5 in Fig. 2). The cross-links of dimers generated by L,D-transpeptidation exclusively contained D-iAsp whereas D-iAsp or L-Ala was found in cross-links generated by D,D-transpeptidation. Thus, Ldt_{fm} tolerated the substitution only in the donor substrate in contrast to the PBPs that catalyzed peptidoglycan cross-linking with modified donor and acceptor substrates. Modifications of the side chain of peptidoglycan precursors were also shown to be tolerated by PBPs of *E. faecalis* and *S. aureus* in previous studies (12,24).

Ldt_{fm} Is Also Specific for D-iAsn-substituted Acceptors in Vitro—The specificity of Ldt_{fm} was analyzed by incubating the enzyme with three disaccharide-tetrapeptides obtained by digestion of peptidoglycan of three different chemotypes with muramidases. The substrates were representative of the main variations found at the third position of peptidoglycan precursors of Gram-positive bacteria including the absence of

a side chain (*meso*DAP³ in *B. subtilis*) and presence of a side chain consisting of D and L amino acids (L-Lys³-D-iAsn in *E. faecium* and L-Lys³-L-Ala-L-Ala in *E. faecalis*) (Fig. 1A). With these three substrates, the cross-linking reaction can potentially lead to the formation of nine dimers including three homodimers, if the same disaccharide-peptide is used as the donor and the acceptor substrate, and six heterodimers, if different disaccharide-peptides are used in all possible combinations (Table 1). Mass spectrometry analyses of the reaction products indicated that Ldt_{fm} catalyzed formation of a homodimer containing D-iAsn-substituted acceptor and donor stem peptides and of a heterodimer containing stem peptides substituted by L-Ala-L-Ala and D-iAsn (Fig. 3). Tandem mass spectrometry was performed to determine whether the L-Ala-L-Ala-substituted disaccharide-peptide had been used as a donor or an acceptor substrate in the formation of the heterodimer (Fig. 4). Fragmentation was performed on lactoyl-peptides obtained by cleavage of the disaccharide-peptides by alkaline treatment since amino acid sequencing of peptidoglycan dimers is more efficient in the absence of the disaccharide moiety of the molecules (24). This treatment also converts D-iAsn into D-iAsp (24). As detailed in Fig. 4, fragmentation of the heterodimer allowed assigning L-Ala-L-Ala to the free side chain of the donor stem and D-iAsp to the cross-link. Thus, Ldt_{fm} tolerated presence of L-Ala in the donor but not in the acceptor substrate of the cross-linking reaction. The specificity of Ldt_{fm} observed *in vitro* in the absence of any other cell wall biosynthesis enzymes accounts for the *in vivo* selection of the acceptor and donor substrates in the derivative *E. faecium* M512 producing the BppA1 transferase (above).

Of note, interaction of the D,D-transpeptidases (PBPs) with their donor and acceptor substrate has not been extensively investigated with purified PBPs, since the enzymes are generally inactive *in vitro* (29), except in very special cases involving highly reactive substrates (e.g. thioester; ref. (30)) and atypical enzymes (e.g. the soluble R61 D,D-peptidase from *Streptomyces* spp. ref. (31,32)). Recently, a peptidoglycan polymerization assay has been developed for the purified PBP1a and

1b of *E. coli* as these bi-functional enzymes catalyze transglycosylation and transpeptidation of the natural substrate, a disaccharide-peptide linked to undecaprenyl lipid carrier by a phosphodiester bond (lipid II) (33,34). This approach has not been yet developed for the PBPs of Gram-positive bacteria that use even more complex precursors due to the presence of an additional side chain. Thus, characterization of Ldt_{fm} in the current study represents the first direct *in vitro* assessment of the substrate specificity of peptidoglycan cross-linking enzyme.

The Ldt_{fm} Homologue from B. subtilis Is Specific for mesoDAP-Containing Acceptors but Functions with Various Donors—Having shown that Ldt_{fm} is specific for its cognate acceptor both *in vivo* and *in vitro*, our following aim has been to determine whether Ldt_{fm} homologues from bacteria producing peptidoglycan of different chemotypes are also specific for their respective acceptor. We have started this analysis with the homologue from *B. subtilis*, Ldt_{Bs}, because the crystal structure of this protein has been recently solved in the framework of a structural genomics project that did not include functional investigations (21). The full length Ldt_{Bs} (167 residues) contains a putative peptidoglycan-binding N-terminal domain consisting of a single LysM module (residues 5-49, Pfam PF01476) and a C-terminal domain that can be superimposed to the catalytic domain of Ldt_{fm} with a root mean square deviation of 1.21Å for the 103 C^α atoms in common (35). The purified protein produced in *E. coli* catalyzed formation of homodimers from disaccharide-tetrapeptides containing *meso*DAP at the third position of the stem peptides (Fig. 5A). Thus, Ldt_{fm} and Ldt_{Bs} displayed both L,D-transpeptidase activity on muropeptides despite limited sequence identity (23 % for the catalytic domain) and different domain compositions (Fig. 1C). Ldt_{Bs} also catalyzed formation of heterodimers containing *meso*DAP³ in one stem and L-Lys³-D-iAsn or L-Lys³-L-Ala-L-Ala in the other stem (Fig. 5A). Tandem mass spectrometry indicated that stems containing L-Lys³-D-iAsn and L-Lys³-L-Ala-L-Ala were present in the donor position of the dimers (Fig. 5B). Thus, Ldt_{Bs} was specific for its cognate *meso*DAP-containing acceptor but

tolerated variations in the structure of the donor substrate.

Ldt_{fs} from E. faecalis Is Specific for Disaccharide-Peptides Containing an L-Ala-L-Ala Side Chain in both the Acceptor and Donor Substrates—The chromosome of *E. faecalis* encodes a protein of 474 residues, designated Ldt_{fs}, which is closely related to Ldt_{fm} (37% identity for the catalytic domain). The two proteins display the same domain composition with an overall sequence identity of 29% (Fig. 1C). Ldt_{fs} catalyzed formation of homodimers from the cognate branched disaccharide-tetrapeptide containing L-Lys³-L-Ala-L-Ala (observed monoisotopic mass of 1,987.96, see Table 1 for the calculated mass). Muropeptides containing *meso*DAP³ or L-Lys³-D-iAsn were not used for dimer formation indicating that Ldt_{fs} is specific for the L-Ala-L-Ala side chain both in the donor and acceptor positions.

Ldt_{fs} Displays L,D-Carboxypeptidase Activity—MS analysis of the product of the L,D-transpeptidation reaction catalyzed by Ldt_{fs} revealed the presence of an additional product (observed monoisotopic mass of 1,916.81) differing from the expected dimer (1987,97; above) by the loss of one alanyl residue (Fig. 6A). Tandem mass spectrometry indicated that this additional product was a dimer generated by L,D-transpeptidation which lacked D-Ala⁴ in the acceptor stem (data not shown). The L,D-carboxypeptidase activity of Ldt_{fs} could have cleaved the L-Lys³-D-Ala⁴ peptide bond in the acceptor stem of the dimer. In addition or alternatively, Ldt_{fs} could have cleaved the substrate prior to its utilisation as an acceptor in the cross-linking reaction since a monomer containing a tripeptide stem ending in L-Lys³ was also detected in the reaction mixture. The latter observation indicated that the L,D-transpeptidase and L,D-carboxypeptidase activities of Ldt_{fs} acted in competition since tripeptide stems cannot be used as a donor substrate. In contrast, L,D-carboxypeptidase activity was not detected for Ldt_{fm} from *E. faecium* (18) and Ldt_{Bs} from *B. subtilis* (data not shown). L,D-carboxypeptidases specific for the L-Lys³-D-Ala⁴ or *meso*DAP³-D-Ala⁴ bond of peptidoglycan precursors have been described in *E. coli* (36), *Pseudomonas aeruginosa* (37), and *Lactococcus lactis* (38). These enzymes are

unrelated to Ldt_{fm} and were only shown to have a hydrolytic activity.

Muropeptides Containing a Stem Pentapeptide are Substrate of Ldt_{fs}—Replacement of the disaccharide-tetrapeptide substituted by L-Ala-L-Ala by the corresponding pentapeptide in the cross-linking assay led to the formation of hydrolysis and transpeptidation products (Fig. 6B). Ldt_{fs} displayed endopeptidase activity since the enzyme cleaved the L-Lys³-D-Ala⁴ peptide bond of the pentapeptide generating a tripeptide and the dipeptide D-Ala-D-Ala. Ldt_{fs} also formed dimers containing a tripeptide stem and a pentapeptide stem at the donor and acceptor position, respectively. Combination of the L,D-transpeptidase and L,D-endopeptidase activity led to the formation of dimers containing two tripeptide stems. In contrast to Ldt_{fs}, the L,D-transpeptidase from *E. faecium* functions exclusively with acyl donors containing a tetrapeptide stem (18).

Exchange Reactions Catalyzed by Ldt_{fs}—The L,D-transpeptidase of *E. faecium* has been previously shown to cleave the L-Lys³-D-Ala⁴ peptide bond of an acyl donor substrate and to form a peptide bond between the alpha carboxyl of L-Lys³ and free D-amino acids (18). Dipeptides were tested in a similar exchange reaction catalyzed by Ldt_{fs} since this enzyme catalyzed formation of tripeptide and D-Ala-D-Ala from pentapeptide (above). Ldt_{fs} used the cognate disaccharide-tetrapeptide and D-Ala-D-Ala as acyl donor and acceptor, respectively, leading to the formation of the corresponding pentapeptide (Fig. 6C). Strikingly, L-Ala-L-Ala was not used as an acyl acceptor although the dipeptide mimics the side chain of the acceptor of the cross-linking reaction. This observation strongly suggests that the dipeptide D-Ala-D-Ala and the L-Ala-L-Ala side chain of muropeptides occupy different subsites in the catalytic cavity of the enzyme. The dipeptide D-Ala-D-Ala may occupy the position of the leaving group of the donor substrate. This would account for the specificity of Ldt_{fs} for D-Ala-D-Ala in the exchange reaction. A distinct subsite may

accommodate the L-Ala-L-Ala side chain of the peptidoglycan precursors. Since the dipeptide L-Ala-L-Ala was not a substrate of Ldt_{fs}, recognition of the acceptor of the cross-linking reaction appears to involve a portion of the molecule larger than the L-Ala-L-Ala moiety of the molecule. The existence of two subsites in the catalytic cavity of the L,D-transpeptidases is supported by the structure of Ldt_{fm} from *E. faecium* (35). The catalytic domain of this enzyme contains two access paths to the putative active cystein residue that could correspond to the binding sites of the acceptor and donor substrates (35).

Conclusions—By-pass of the D,D-transpeptidase activity of the PBPs by the L,D-transpeptidase activity of Ldt_{fm} confers high-level cross-resistance to β -lactams and glycopeptides in *E. faecium* since these drugs do not interact with the enzyme and its substrate, respectively (9). Ldt_{fm} is the first functionally characterized member of a novel family of cystein peptidases which are widespread both in Gram-positive and Gram-negative bacteria (18,35). We have characterized two additional members of the family from *B. subtilis* (Ldt_{Bs}) and *E. faecalis* (Ldt_{fs}) and shown that these enzymes catalyze the cross-linking of purified peptidoglycan fragments *in vitro*. Comparison of the specificity of Ldt_{fm}, Ldt_{Bs}, and Ldt_{fs} (Table 2) indicated that diversification of the structure of peptidoglycan precursors associated with speciation had led to a parallel evolution of the substrate specificity of members of the Ldt_{fm} protein family. This evolution concern mainly the acceptor since for this substrate all three L,D-transpeptidases were specific for their cognate disaccharide-peptides. In contrast, Ldt_{fm} and Ldt_{Bs} tolerated substitutions at the third position of the donor. The specificity of the L,D-transpeptidases for the acceptor stem indicates that blocking the assembly of the side chain of peptidoglycan precursors is a potential strategy to combat resistance involving L,D-transpeptidases.

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FOOTNOTES

The authors thank L. Gutmann for helpful comments on the manuscript. The abbreviations used are: D-iAsn, D-*iso*-asparagine; D-iAsp, D-*iso*-aspartic acid; D-iAsx, D-iAsn or D-iAsp; GlcNAc, *N*-acetylglucosamine; *meso*DAP, *meso*-diaminopimelic acid; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MurNAc, *N*-acetylmuramic acid; PBP, penicillin-binding protein; rp-HPLC, reverse-phase high-pressure liquid chromatography.

FIGURE LEGENDS

FIGURE 1. Diversity of the structure of peptidoglycan cross-links and their mode of synthesis in Gram-positive bacteria. (A) Structure of peptidoglycan subunits from *E. faecium*, *E. faecalis*, and *B. subtilis*. The disaccharide-peptide subunit contains β -1-4 linked *N*-acetyl-glucosamine (GlcNAc) and *N*-acetyl-muramic acid (MurNAc). The D-lactoyl moiety of MurNAc is substituted by a stem pentapeptide containing L-Lys³ in *E. faecium* and *E. faecalis* or *meso*-diaminopimelic acid (*meso*DAP³) in *B. subtilis* and *E. coli*. The side chain amino group of L-Lys³ is substituted by a D-*iso*-asparaginyl or D-*iso*-aspartyl residue (D-iAsx) in *E. faecium* and by L-Ala-L-Ala in *E. faecalis*. The amino group of the acyl acceptor participating in the formation of the cross-links is indicated by an arrow. (B) Schematic representation of muropeptide dimers generated by D,D- and L,D-transpeptidation. The acceptor stem in the represented dimers is a tripeptide ending in L-Lys³. Additional residues can be found at its position (D-Ala⁴, Gly⁴, D-Ala⁴-D-Ala⁵) (9). (C) Domain composition of L,D-transpeptidases from *E. faecium* (Ldt_{fm}), *E. faecalis* (Ldt_{fs}), and *B. subtilis* (Ldt_{Bs}). Hatched boxes represent hydrophobic regions that could act as membrane anchors in Ldt_{fm} and Ldt_{fs} (35). Ldt_{Bs} comprises a LysM peptidoglycan-binding module linked to the catalytic domain (domain II) (21). For each L,D-transpeptidase, the portion of the proteins that has been

produced in *E. coli* is indicated by a thick line terminated by the sequence of the affinity tag (one letter code).

FIGURE 2. Peptidoglycan composition of *E. faecium* M512 producing BppA1 from *E. faecalis*. (A) rp-HPLC profile of mucopeptides from *E. faecium* M512/pJEH6(*bppA1*). Bacteria were grown in the presence of spectinomycin (120 $\mu\text{g/ml}$) to counter select loss of plasmid pJEH6(*bppA1*) and of isopropyl- β -D-thiogalactopyranoside (0.3 mM) to induce the *bppA1* gene encoding a transferase of the Fem family for incorporation of L-Ala into the side chain of peptidoglycan precursors. Peptidoglycan was extracted with SDS, treated with ammonium hydroxide, and the resulting lactoyl-peptides were separated by rp-HPLC. mAU, absorbance unit $\times 10^3$ at 210 nm. (B) Structure of mucopeptides. The relative abundance (%) of the material in peaks 1 to 5 was calculated by integration of the absorbance at 210 nm. The retention time (RT) is indicated for minor mucopeptides which could not be assigned to specific peaks due to their low abundance. The structure of lactoyl-peptides was deduced from the observed monoisotopic mass (Mass) and confirmed by tandem mass spectrometry for most monomers and dimers (indicated by a star). The observed and calculated mass differed at the maximum by 0.2. The stem peptide of monomers and the acceptor stem of dimers consisted of the tripeptide L-Ala¹-D-iGln²-L-Lys³ (Tri), the tetrapeptide L-Ala¹-D-iGln²-L-Lys³-D-Ala⁴ (Tetra), and the pentapeptide L-Ala¹-D-iGln²-L-Lys³-D-Ala⁴-D-Ala⁵ (Penta). In certain mucopeptides of low abundance, the C-terminal D-Ala⁴ was replaced by a glycyl residue (Gly C-ter). Cross-links generated by D,D-tanspeptidases (DD) contained D-iAsp or L-Ala (D-Ala⁴ \rightarrow D-iAsp-L-Lys³ and D-Ala⁴ \rightarrow L-Ala-L-Lys³). Cross-links generated by L,D-transpeptidation (LD) exclusively contained D-iAsp (L-Lys³ \rightarrow D-iAsp-L-Lys³).

FIGURE 3. Analysis of the products of the cross-linking reaction catalyzed by Ldt_{fm} in vitro. Three reduced disaccharide-tetrapeptides containing *meso*DAP³, L-Lys³-D-iAsn, or L-Lys³-L-Ala-L-Ala were incubated with Ldt_{fm} and the products of the cross-linking reaction were analyzed by nanoelectrospray mass spectrometry. Peaks at m/z 942.38, 1,011.41, and 1,039.46 were assigned to be the $[M+H]^+$ ions of the substrates (reduced disaccharide-tetrapeptides containing *meso*DAP³, L-Lys³-D-iAsn, or L-Lys³-L-Ala-L-Ala, respectively). Peaks at m/z 966.93, 977.89, and 985.88 were assigned to be the $[M+2H]^{2+}$, $[M+H+Na]^{2+}$, and $[M+H+K]^{2+}$ ions of the homodimer containing L-Lys³-D-iAsn in the donor and acceptor stems as the deduced monoisotopic mass (1,931.85) matched the calculated mass (1,931.91; Table 1). The monoisotopic mass of 1,959.82, deduced from peaks at m/z 980.93 $[M+2H]^{2+}$, 991.91 $[M+H+Na]^{2+}$, 999.89 $[M+H+K]^{2+}$, matched the calculated monoisotopic mass of a heterodimer containing L-Lys³-D-iAsn and L-Lys³-L-Ala-L-Ala (1,959.94). Minor peaks at m/z 964.74 and 974.06 were assigned to be the $[M+2H+K]^{3+}$ ions of trimers containing three L-Lys³-D-iAsn (calculated monoisotopic mass of 2,853.33) or two L-Lys³-D-iAsn and one L-Lys³-L-Ala-L-Ala (2,881.36), respectively.

FIGURE 4. Structure of the heterodimer formed by Ldt_{fm}. Fragmentation was performed on the ion at m/z 1,145.59 obtained by ammonium hydroxide treatment of the heterodimer with a monoisotopic mass of 1,959.82 (Table 1). Boxes indicate ions generated by cleavage at single peptide bonds as indicated in the structure of the dimer. Ions at m/z 604.23 and 1,003.51 labelled with arrows establish that D-iAsp is present in the cross-link whereas L-Ala-L-Ala is located in the free side chain of the donor stem. The other peaks could correspond to additional loss of NH₃ and CO plus NH₃ and to combinations of fragmentations at two peptide bonds, as previously described (24). D-Lac, D-lactoyl.

FIGURE 5. Analysis of the dimers formed by Ldt_{Bs}. (A) MS analysis of the products of the cross-linking reaction catalyzed by Ldt_{Bs}. Three reduced disaccharide-tetrapeptides containing *meso*DAP³, L-Lys³-D-iAsn, or L-Lys³-L-Ala-L-Ala were incubated with Ldt_{Bs} and the products of the reaction were analyzed by nanoelectrospray MS. Peaks at m/z 942.41, 964.39, and 980.36 were assigned to be the $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions of the substrate containing *meso*DAP³, respectively. Peaks at m/z 897.92, 908.90, 916.89, 919.88, 927.88, and 935.86 were assigned to be the $[M+H+H]^{2+}$, $[M+H+Na]^{2+}$, $[M+H+K]^{2+}$, $[M+Na+Na]^{2+}$, $[M+Na+K]^{2+}$, and $[M+K+K]^{2+}$ ions of the homodimer containing *meso*DAP³ in the donor

and acceptor stems (calculated monoisotopic mass of 1,793.77; Table 1). The monoisotopic mass of 1,862.68 deduced from peaks at m/z 932.44 $[M+2H]^{2+}$, 943.43 $[M+H+Na]^{2+}$, 951.42 $[M+H+K]^{2+}$, 954.43 $[M+Na+Na]^{2+}$, and 962.41 $[M+Na+K]^{2+}$ matched the calculated monoisotopic mass of a heterodimer containing *meso*DAP³ and L-Lys³-D-iAsn (1,862.84). The monoisotopic mass of 1,890.67 deduced from peaks at m/z 946.45 $[M+2H]^{2+}$, 957.45 $[M+H+Na]^{2+}$, and 965.43 $[M+H+K]^{2+}$ matched the calculated monoisotopic mass of a heterodimer containing *meso*DAP³ and L-Lys³-L-Ala-L-Ala (1,890.87). (B) Fragmentation of dimers obtained by ammonium hydroxide treatment. The peaks generated by the cleavage of a single peptide bond are boxed. Arrows indicate peaks used to assign stem peptides to the acceptor and donor positions of heterodimers.

FIGURE 6. Diversity of the reactions catalyzed by Ldt_{fs} *in vitro*. (A) Products generated from a disaccharide-tetrapeptide by the L,D-carboxypeptidase and the L,D-transpeptidase activities of Ldt_{fs} alone or in combination. (B) Products generated from a disaccharide-pentapeptide by the L,D-endopeptidase and L,D-transpeptidase activities of Ldt_{fs}. (C) Use of the dipeptide D-Ala-D-Ala as an acyl acceptor to form a pentapeptide from a tetrapeptide.

TABLE 1. Calculated monoisotopic mass of the dimers generated by L,D-transpeptidation. Cross-linking of three reduced disaccharide-tetrapeptides containing L-Lys³-D-iAsn, *meso*DAP³, and L-Lys³-L-Ala-L-Ala in all possible combinations can lead to a total of nine dimers including three homodimers and six heterodimers. The calculated monoisotopic mass of the corresponding lactoyl-peptides is indicated in parenthesis. Disaccharide-peptides and lactoyl-peptides contain D-iAsn and D-iAsp, respectively.

Third position of the tripeptide donor stem (X ³ -side chain)	Third position of the tetrapeptide donor stem (X ³ -side chain)		
	L-Lys ³ -D-iAsx	<i>meso</i> DAP ³	L-Lys ³ -L-Ala- L-Ala
L-Lys ³ -D-iAsx	1,931.91 (1,117.53)	1,862.84 (1,047.47)	1,959.94 (1,144.57)
<i>meso</i> DAP ³	1,862.84 (1,047.47)	1,793.77 (977.42)	1,890.87 (1,074.52)
L-Lys ³ -L-Ala- L-Ala	1,959.94 (1,144.57)	1,890.87 (1,074.52)	1,987.97 (1,171.62)

TABLE 2. Muropeptides used as acceptor and donor substrates by the L,D-transpeptidases from *E. faecium* (Ldt_{fm}), *E. faecalis* (Ldt_{fs}) and *B. subtilis* (Ldt_{Bs}). The substrates were disaccharide-tetrapeptides differing at the third position as indicated (X³-side chain).

L,D-transpeptidase	Acceptor	Donor
Ldt _{fm}	L-Lys ³ -D-iAsn	L-Lys ³ -D-iAsn L-Lys ³ -L-Ala-L-Ala
Ldt _{Bs}	<i>meso</i> DAP ³	<i>meso</i> DAP ³ L-Lys ³ -D-iAsn L-Lys ³ -L-Ala-L-Ala
Ldt _{fs}	L-Lys ³ -L-Ala-L-Ala	L-Lys ³ -L-Ala-L-Ala

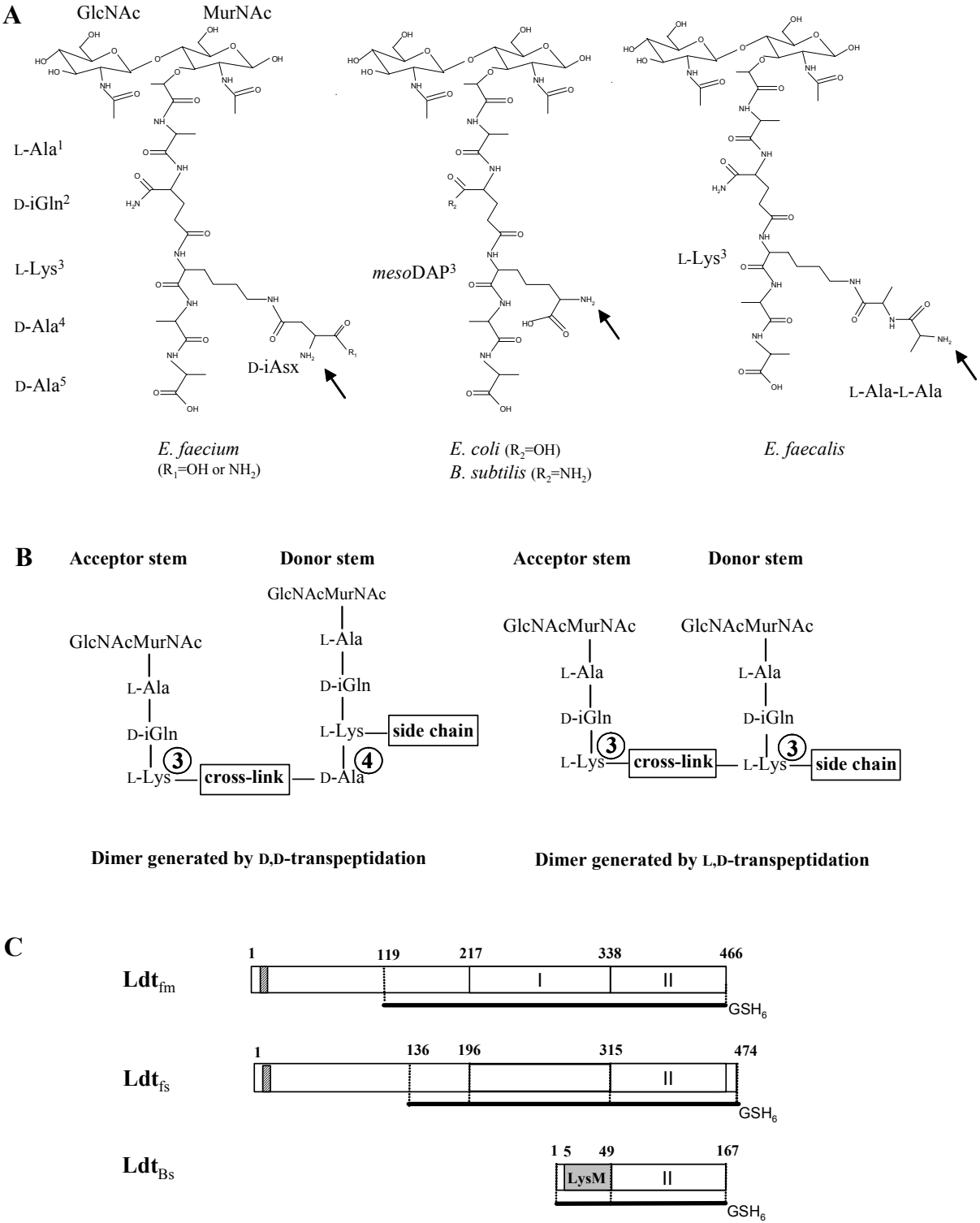
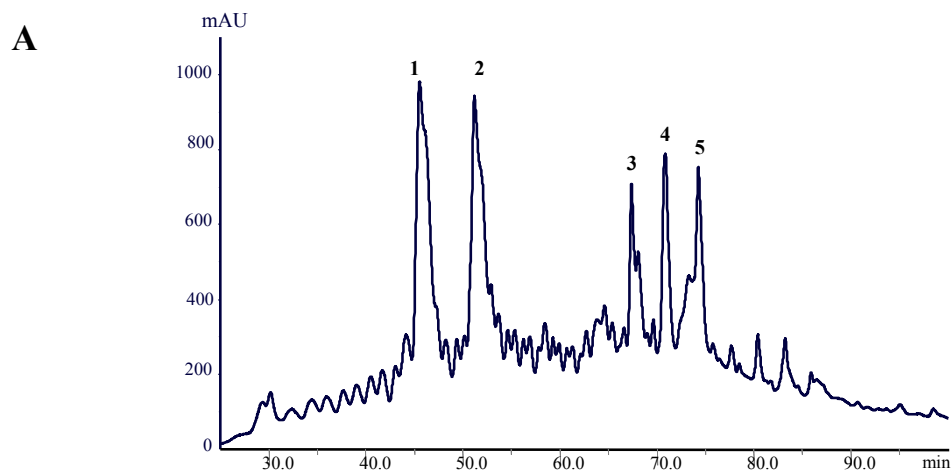


Fig. 1



B

Major monomers (57.8%)					Major Dimers (42.2%)																																						
Peak	%	Mass	Stem	Side chain	Peak	%	Mass	Acceptor Stem	Cross-link		Side chain																																
1	32.5	488.2*	Tri	L-Ala	3	11.9	1,073.5* 1,073.5*	Tri Tetra	DD LD	D-iAsp D-iAsp	L-Ala L-Ala																																
2	25.3	559.3*	Tetra	L-Ala	4	11.7	1,029.5* 1,144.6*	Tri Tetra	DD DD	L-Ala D-iAsp	L-Ala L-Ala																																
Minor monomers <table border="1"> <thead> <tr> <th>Mass</th> <th>RT</th> <th>Stem</th> <th>Side chain</th> </tr> </thead> <tbody> <tr> <td>417.2*</td> <td>30.2</td> <td>Tri</td> <td>none</td> </tr> <tr> <td>474.2*</td> <td>32.5</td> <td>Tetra (Gly C-ter)</td> <td>none</td> </tr> <tr> <td>488.3*</td> <td>39.0</td> <td>Tetra</td> <td>none</td> </tr> <tr> <td>532.2*</td> <td>42.0</td> <td>Tri</td> <td>D-iAsp</td> </tr> <tr> <td>603.3</td> <td>43.0</td> <td>Tetra</td> <td>D-iAsp</td> </tr> <tr> <td>545.3*</td> <td>45.6</td> <td>Tetra (Gly C-ter)</td> <td>L-Ala</td> </tr> <tr> <td>630.3*</td> <td>54.6</td> <td>Penta</td> <td>L-Ala</td> </tr> </tbody> </table>					Mass	RT	Stem	Side chain	417.2*	30.2	Tri	none	474.2*	32.5	Tetra (Gly C-ter)	none	488.3*	39.0	Tetra	none	532.2*	42.0	Tri	D-iAsp	603.3	43.0	Tetra	D-iAsp	545.3*	45.6	Tetra (Gly C-ter)	L-Ala	630.3*	54.6	Penta	L-Ala	5	18.6	1,100.6*	Tetra	DD	L-Ala	L-Ala
					Mass	RT	Stem	Side chain																																			
					417.2*	30.2	Tri	none																																			
					474.2*	32.5	Tetra (Gly C-ter)	none																																			
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Minor Dimers <table border="1"> <thead> <tr> <th>Mass</th> <th>RT</th> <th>Acceptor Stem</th> <th colspan="2">Cross-link</th> <th>Side chain</th> </tr> </thead> <tbody> <tr> <td>1,117.5 1,059.5*</td> <td>63.7 63.7</td> <td>Tetra Tetra (Gly C-ter)</td> <td>LD LD</td> <td>D-iAsp D-iAsp</td> <td>D-iAsp L-Ala</td> </tr> <tr> <td>1,002.5*</td> <td>64.6</td> <td>Tri</td> <td>LD</td> <td>D-iAsp</td> <td>L-Ala</td> </tr> <tr> <td>1,130.6*</td> <td>66.0</td> <td>Tetra (Gly C-ter)</td> <td>DD</td> <td>D-iAsp</td> <td>L-Ala</td> </tr> </tbody> </table>					Mass	RT	Acceptor Stem	Cross-link		Side chain	1,117.5 1,059.5*	63.7 63.7	Tetra Tetra (Gly C-ter)	LD LD	D-iAsp D-iAsp	D-iAsp L-Ala	1,002.5*	64.6	Tri	LD	D-iAsp	L-Ala	1,130.6*	66.0	Tetra (Gly C-ter)	DD	D-iAsp	L-Ala															
					Mass	RT	Acceptor Stem	Cross-link		Side chain																																	
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					1,002.5*	64.6	Tri	LD	D-iAsp	L-Ala																																	
1,130.6*	66.0	Tetra (Gly C-ter)	DD	D-iAsp	L-Ala																																						

Fig. 2

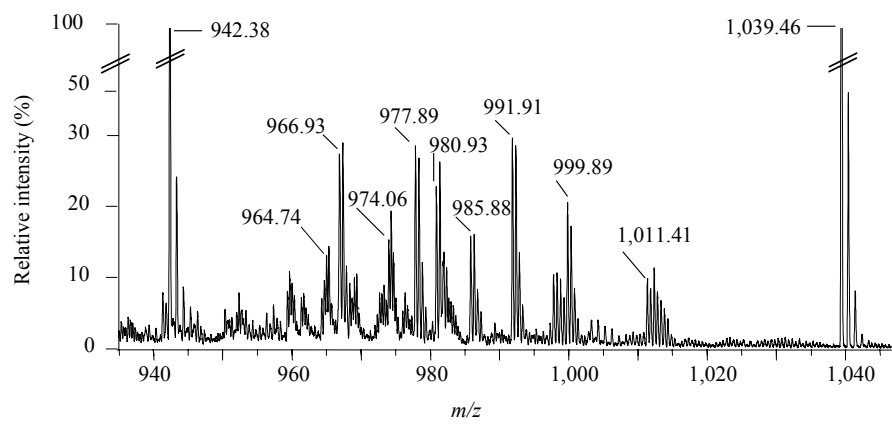


Fig. 3

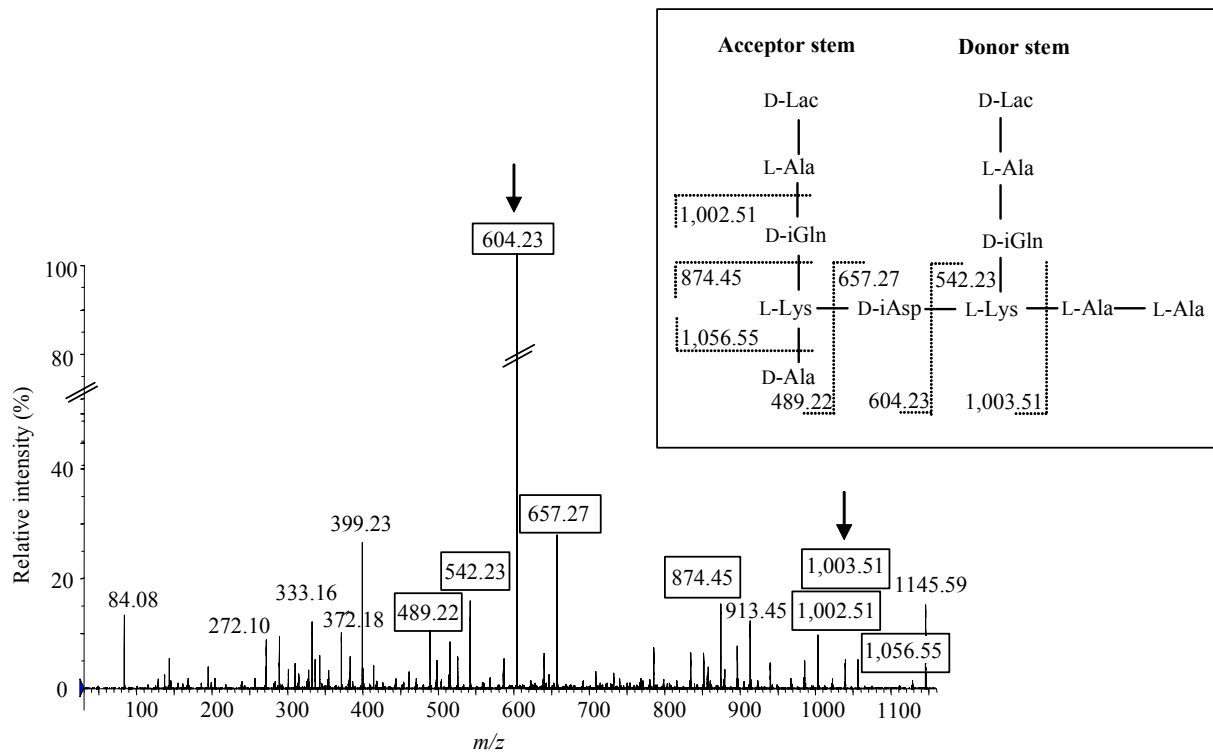


Fig. 4

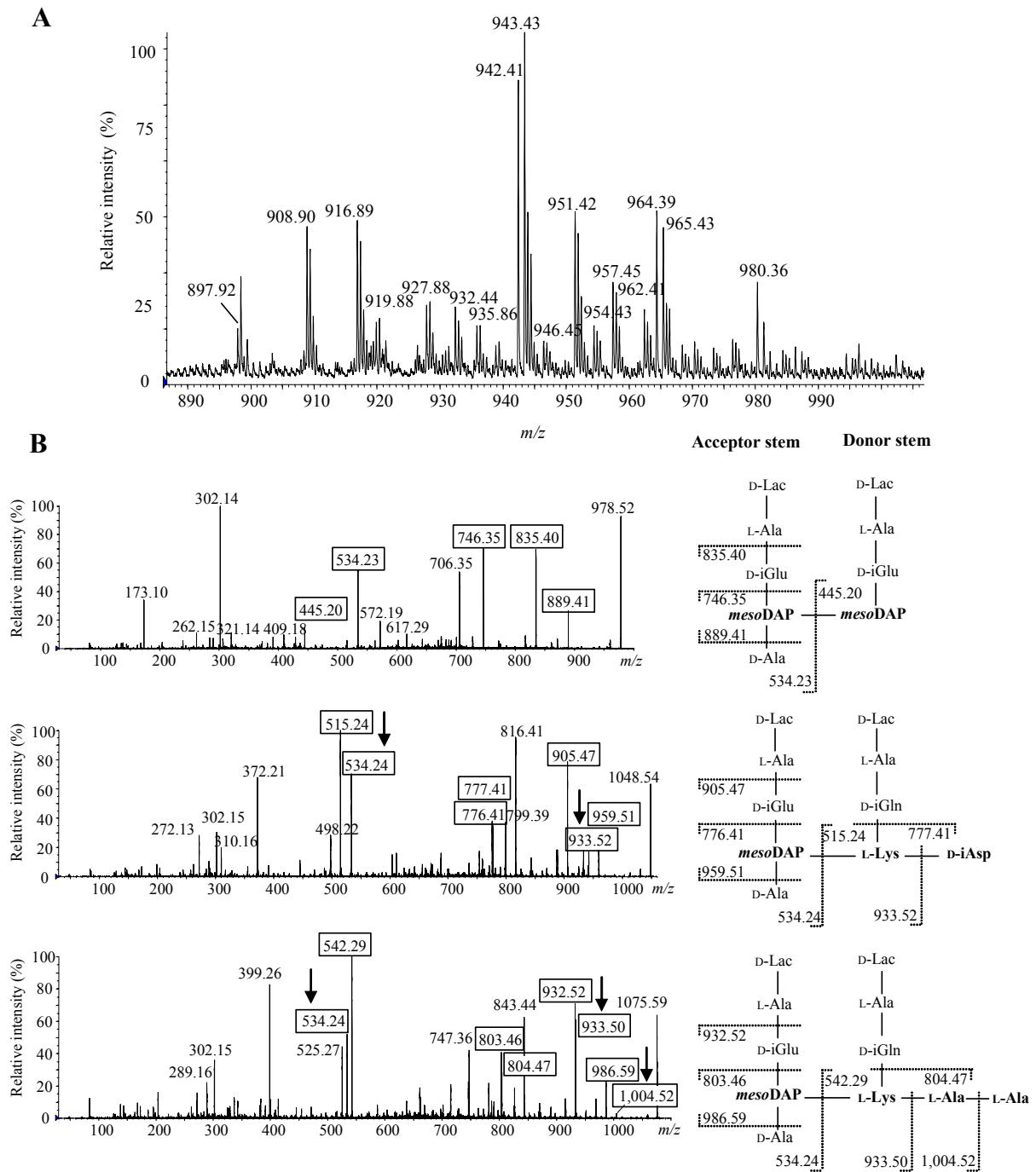


Fig. 5

A

Substrate	Products		
GlcNAcMurNAc L-Ala D-iGln L-Lys- L-Ala - L-Ala D-Ala	GlcNAcMurNAc L-Ala D-iGln L-Lys- L-Ala - L-Ala (+ D-Ala) L,D-carboxypeptidase	GlcNAcMurNAc GlcNAcMurNAc L-Ala L-Ala D-iGln D-iGln L-Lys- L-Ala - L-Ala - L-Lys- L-Ala - L-Ala D-Ala (+ D-Ala) L,D-transpeptidase	GlcNAcMurNAc GlcNAcMurNAc L-Ala L-Ala D-iGln D-iGln L-Lys- L-Ala - L-Ala - L-Lys- L-Ala - L-Ala (+ 2 D-Ala) L,D-transpeptidase and L,D-carboxypeptidase

B

Substrate	Products		
GlcNAcMurNAc L-Ala D-iGln L-Lys- L-Ala - L-Ala D-Ala D-Ala	GlcNAcMurNAc L-Ala D-iGln L-Lys- L-Ala - L-Ala (+ D-Ala-D-Ala) L,D-endopeptidase	GlcNAcMurNAc GlcNAcMurNAc L-Ala L-Ala D-iGln D-iGln L-Lys- L-Ala - L-Ala - L-Lys- L-Ala - L-Ala D-Ala D-Ala (+ D-Ala-D-Ala) L,D-transpeptidase	GlcNAcMurNAc GlcNAcMurNAc L-Ala L-Ala D-iGln D-iGln L-Lys- L-Ala - L-Ala - L-Lys- L-Ala - L-Ala (+ 2 D-Ala-D-Ala) L,D-transpeptidase and L,D-endopeptidase

C

Substrates	Products
GlcNAcMurNAc L-Ala D-iGln L-Lys- L-Ala - L-Ala D-Ala	GlcNAcMurNAc L-Ala D-iGln L-Lys- L-Ala - L-Ala D-Ala D-Ala (+ D-Ala)
D-Ala D-Ala	

Fig. 6