A Novel Peptidoglycan Cross-linking Enzyme for a β-Lactam-resistant Transpeptidation Pathway*

The β-lactam antibiotics remain the most commonly used to treat severe infections. Because of structural similarity between the β-lactam ring and the D-alanyl-D-alanine extremity of bacterial cell wall precursors, the drugs act as suicide substrates of the DD-transpeptidases that catalyze the last cross-linking step of cell wall assembly. Here, we show that this mechanism of action can be defeated by a novel type of transpeptidase identified for the first time by reverse genetics in a β-lactam-resistant mutant of Enterococcus faecium. The enzyme, Ldtfm, catalyzes in vitro the cross-linking of peptidoglycan subunits in a β-lactam-insensitive LD-transpeptidation reaction. The specificity of Ldtfm for the L-lysyl-D-alanine peptide bond of tetrapeptide donors accounts for resistance because this substrate does not mimic β-lactams in contrast to D-alanyl-D-alanine in the pentapeptide donors required for DD-transpeptidation. Ldtfm homologues are encountered sporadically among taxonomically distant bacteria, indicating that LD-transpeptidase-mediated resistance may emerge in various pathogens.

More than 60 years after the introduction of penicillin, the class of β-lactam antibiotics remains the most commonly used to treat severe infections despite the emergence of various resistance mechanisms, including drug detoxification by production of β-lactamases (1), decreased affinity of the target (2), and decreased permeability (3). The success story of β-lactams involves the development of several generations of β-lactams to defeat the resistance mechanisms and their association with inhibitors of β-lactamases (4, 5). All β-lactams have a common mechanism of action, as they share structural similarity with bacterial cell wall precursors. These drugs act as suicide substrates of the DD-transpeptidase catalytic domain of the penicillin-binding proteins (PBPs) responsible for the last cross-linking step of cell wall assembly (Fig. 1A) (2, 6, 7).

Among the DD-transpeptidases of Enterococcus faecium, the low affinity PBPs is unessential for growth but responsible for intrinsic low level resistance to ampicillin (8, 9). The first-line drug used at high dosage to treat severe enterococcal infections. In clinical isolates, higher levels of resistance to ampicillin are commonly associated with multiple amino acid substitutions in the transpeptidase domain of PBPs (10, 11). We have shown previously that even higher levels of resistance to ampicillin can be achieved by a novel mechanism in the absence of PBPs (12). Analysis of the peptidoglycan of the in vitro selected β-lactam-resistant mutant E. faecium M512 revealed that the D-Ala4→D-iAsn-L-Lys3 cross-link had been replaced by a novel type of cross-link between the L-Lys3 carboxyl group of a donor and the side-chain D-iAsn of an acceptor (12) (Fig. 1B). In the presence of ampicillin, the novel L-Lys3→D-iAsn-L-Lys3 cross-links were exclusively found in the peptidoglycan of E. faecium M512, indicating that this DD-transpeptidase activity of the PBPs did not participate in the formation of the cross-links. Selection of E. faecium M512 from E. faecium D344S in five consecutive steps on increasing concentrations of ampicillin led to the gradual activation of the LD-transpeptidation pathway because the proportion of L-Lys3→D-iAsn-L-Lys3 was 3.1% for D344S and increased at each selection step (13). Activation of the resistance pathway was associated with the production of a DD-carboxypeptidase that generated precursors containing a tetrapeptide stem in the cytoplasm of E. faecium M512 (13). Based on these observations, we have speculated that precursors containing a tetrapeptide stem are essential for the formation of the L-Lys3→D-iAsn-L-Lys3 cross-links.

In the present work, we have identified in E. faecium an LD-transpeptidase as the key enzyme of this alternate transpeptidation pathway and have shown that its substrate does not bear structural similarity to β-lactams. The bypass mechanism could invalidate the β-lactams as the major class of antibiotics.

**EXPERIMENTAL PROCEDURES**

Purification of the LD-Transpeptidase and N-terminal Sequencing—
The LD-transpeptidase from E. faecium (Ldtfm) was partially purified in four chromatographic steps using a radioactive exchange assay (13) to detect active fractions. Briefly, E. faecium M512 (12) was grown to an A600 of 0.7 in 24 liters of brain heart infusion broth (Difco), harvested by centrifugation, and washed twice in 10 mM sodium phosphate (pH 7.0). Bacteria were disrupted with glass beads in a cell disintegrator (The Mickle Laboratory Engineering Co., Gromshall, UK) for 2 h at 4 °C. The extract was centrifuged (5,000 × g for 10 min at 4 °C) to remove cell
debris, and the supernatant was ultracentrifuged at 100,000 × g for 30 min at 4 °C. Soluble proteins (1 g) were loaded onto an anion exchange column (Hi-Load™ 26/10 Q-Sepharose™ HP, Amersham Biosciences) equilibrated with 25 mM sodium cacodylate buffer (pH 7.86) (buffer A). Elution was performed with a linear 0–2 M NaCl gradient in buffer A. Active fractions were pooled (30 mg of proteins), concentrated by ultrafiltration (Centricon YM10, Millipore, Saint-Quentin-en-Yvelines, France), and loaded onto a gel filtration column (Superdex 75 HR26/60, Amersham Biosciences) equilibrated with buffer A containing 0.3 M NaCl. Active fractions (1 mg of proteins) were loaded onto a weak anion exchange column (HiTrap™ DEAE fast flow™ 1 ml, Amersham Biosciences) equilibrated with buffer A. Proteins (300 µg) eluting between 0.2 and 0.3 M NaCl were concentrated by ultrafiltration (Amicon ultra-4, Millipore) and loaded onto a gel filtration column (Superdex 200 PC 3.2/30, Amersham Biosciences) equilibrated with buffer A containing 0.3 M NaCl. Active fractions (70 µg of proteins) were concentrated (Amicon ultra-4) and analyzed by SDS-PAGE revealing a major 48-kDa protein band that was transferred onto a polyvinylidene difluoride membrane (Problott, Applied Biosystems, Framingham, MA) by passive adsorption (14). N-terminal Edman sequencing was performed on an Applied Biosystems Procise 494HT instrument with reagents and methods recommended by the manufacturer. The open reading frame for the ldtM open reading frame of E. faecium was identified using the software tBLAST at the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov/). The partially purified 48-kDa protein corresponded to a proteolytic fragment because the sequence encoding its N terminus was not preceded by a translation initiation codon. The upstream sequence contained a single likely translation initiation site (ACTTAaggTTGTCCGATtag), consisting of an ATG codon preceded by a putative ribosome binding site (lowercase).

Production of the LdtM-Transpeptidase in E. coli and Purification of the Protein—A portion of the ldtM open reading frame of E. faecium M512 was amplified with the primers 5'-TTCCATGCAGAAAACAAAGAATAGATCC-3' and 5'-TGGATCCGAGCAATTACAGGCG-3'. The PCR product digested with NcoI and BamHI (underlined) was cloned into pET2818, a derivative of pET2816 (15) lacking the sequence specifying the thrombin cleavage site (our laboratory collection). The resulting plasmid, pET2818fLdtM, was introduced into

![Image](https://example.com/image.png)
E. coli BL21(DE3) harboring pREP4GroESL (16), and bacteria were grown at 37 °C to an A650 of 0.8 in three liters of brain heart infusion broth containing ampicillin (100 μg/ml). Isopropyl D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and incubation was continued for 17 h at 16 °C. Ldtfm was purified from a clarified lysate by affinity chromatography on Ni2+–nitrilotriacetate-agarose resin (Qiagen GmbH, Hilden, Germany) followed by anion exchange chromatography (MonoQ HR5/5, Amersham Biosciences) with a NaCl gradient in Tris-HCl, pH 7.5. An additional gel filtration was performed on a Superdex HR10/30 column (Amersham Biosciences) equilibrated with 50 mM Tris-HCl (pH 7.5) containing 300 mM NaCl at a flow rate of 0.5 ml/min. The overall yield was 3 mg/liter of culture. Site-directed mutagenesis was performed according to the QuikChange procedure of Stratagene (La Jolla, CA).

Substrates of the Ld-Transpeptidase—The dipeptide Nε,Nδ-diacytethyl-l-lysyl-d-alanine (Ac2-L-Lys-D-Ala) was prepared as described previously (13). Nε,Nδ-diacytethyl-l-lysine-d-alanine (Ac2-L-Lys-D-Ala), l-Ala-d-iGlu-l-Lys-d-Ala-d-Ala (pentapeptide), and amino acids were purchased from Sigma. D-2-Hydroxy acids were obtained from Acros Organics (Noisy-Le-Grand, France). UDP-N-acetylmuramyl-l- Ala-d-iGlu-l-Lys-d-Ala-d-Ala (UDP-MurNAc-pentapeptide) was prepared from Staphylococcus aureus (17). The R39 dd-carboxypeptidase (18) was used to generate UDP-MurNAc-L-Ala-d-iGlu-l-Lys-d-Ala (UDP-MurNAc-tetrapeptide) and l-Ala-d-iGlu-l-Lys-d-Ala (tetrapeptide) from UDP-MurNAc pentapeptide and pentapeptide, respectively. Disaccharide peptide fragments of the peptidoglycan (muropeptides) of Enterococcus gallinarum were prepared by scalving up a previously procedure published previously (19) including GlcNAc-MurNAc-L-Ala-d-iGln-l- (Nε-DiAsn) Lys-d-Ala-d-Ala (GlcNAc-MurNAc-pentapeptide-iAsn), GlcNAc-MurNAc-L-Ala-d-iGln-l- (Nε-DiAsn) Lys-d-Ala (GlcNAc-MurNAc-tetrapeptide-iAsn), GlcNAc-MurNAc-L-Ala-d-iGln-l-Lys-d-Ala (GlcNAc-MurNAc-tripeptide-iAsn), and GlcNAc-MurNAc-L-Ala-d-iGln-l-Lys-d-Ala (GlcNAc-MurNAc-tetrapeptide). The concentration of muropeptides was estimated after acidic hydrolysis (20). The structure of the different substrates was confirmed by mass spectrometry and tandem mass spectrometry as described previously (21).

Ld-Transpeptidase Assays—The exchange reaction assays for the capacity of the enzyme to catalyze cleavage of the L-Lys-d-Ala peptide bond of the model donor dipeptide substrate Ac2-L-Lys-D-Ala and formation of a peptide bond between Ac2-L-Lys and d-[14C]Ala (13, 22). The standard assay (50 μl) contained Ac2-L-Lys-d-Ala (5 mM), d-[14C]Ala (0.15 mM) 2.0 GBq/mmol, ICN Pharmaceuticals, Orsay, France), 10 mM sodium cacodylate buffer (pH 6.0), and 0.1% Triton X-100.
X-100 (v/v). The reaction products were separated by reverse phase HPLC and detected by scintillation with a radioflow detector (LB508, PerkinElmer Life Sciences) coupled to the HPLC device (13). To assay for inhibition by ampicillin, Ldt_{fm} (3 μg) was incubated with Ac\_2-L-Lys-D-Ala (0.3 mM), D-\[^{14}C\]Ala (0.15 mM), and various concentrations of the drug. To test different donors, 3 μg of Ldt_{fm} were incubated for 60 min in the same conditions, except that Ac\_2-L-Lys-D-Ala was replaced by UDP-MurNAc-tetrapeptide (2.5 mM), UDP-MurNAc-pentapeptide (2.5 mM), tetrapeptide (2.5 mM), pentapeptide (2.5 mM), GlcNAc-MurNAc-tetrapeptide-iAsn (1 mM), and GlcNAc-MurNAc-pentapeptide-iAsn (1 mM).

To assay for in vitro transpeptidation, the Ld-transpeptidase (3 μg) was incubated with the monomeric muropeptides GlcNAc-MurNAc-L-Ala-D-iGln-L-(N\(^{\alpha}\)-D-iAsn) (25 nmol), GlcNAc-MurNAc-L-Ala-D-iGln-L-(N\(^{\alpha}\)-D-iAsn)Lys (5 nmol), and GlcNAc-MurNAc-L-Ala-D-iGln-L-Lys-D-Ala (5 nmol) for 2 h at 37 °C in 25 μl of 5 mM sodium phosphate buffer (pH 6.0). The reaction was stopped by boiling the sample for 3 min, and the mixture was centrifuged (10,000 × g, 2 min). The formation of dimers was determined by mass spectrometry on a 10-μl aliquot. For tandem mass spectrometry analysis, the remainder of the reaction mixture was treated with ammonium hydroxide to cleave the ether link internal to MurNAc (21). The conditions for fragmentation of the resulting lactoyl peptides with N\(_2\) as the collision gas were as described previously (21).

RESULTS AND DISCUSSION

Identification of the Ld-Transpeptidase of E. faecium M512 (Ldt_{fm})—We identified the gene encoding the Ld-transpeptidase responsible for the formation of the L-Lys\(^{3}\)→D-iAsn-L-Lys\(^{3}\) cross-links in E. faecium M512 by partial purification of the enzyme (48 kDa), sequencing of its N
terminus, and similarity searches in the partial genome sequence of E. faecium (Fig. 2, A and B). The partially purified protein was a proteolytic fragment lacking the 118 N-terminal residues, including a putative membrane anchor. The portion of the open frame encoding the proteolytic fragment was expressed in Escherichia coli for large scale protein purification (Fig. 2C). The protein was active in an exchange assay (Fig. 2D) and was not inhibited by ampicillin (Fig. 2E), indicating that the gene encoding the L,D-transpeptidase of E. faecium M512 (Ldtfm) had been successfully identified.

Specificity of Ldtfm—To gain insight into the activity of Ldtfm, various 2-amino and 2-hydroxy acids were tested as potential acceptor substrates in an exchange reaction using the model dipeptide substrate Ac2-L-Lys-D-Ala as the donor (Fig. 2F and TABLE ONE). Formation of depsipeptides with D-lactic, D-2-hydroxyhexanoic, and D-malic acids revealed that Ldtfm can catalyze formation of ester bonds in addition to peptide bonds. Acceptors containing a relatively bulky side chain such as D-Met and D-2-hydroxyhexanoic acid were used as acceptors in the transpeptidation and transesterification reactions. Hydrolysis of the C-terminal D-Ala of Ac2-L-Lys-D-Ala was not detected in the presence of a suitable acceptor substrate, indicating a biosynthetic function for Ldtfm, in contrast to the previously characterized L,D-carboxypeptidase involved in peptidoglycan recycling in E. coli (23). Finally, Ldtfm was stereo-specific because no product was detected when L-Met was used as donor (TABLE ONE).

The Ldtfm specificity for peptide donors was explored with the exchange assay using D-[14C]Ala as the acceptor. Formation of radioactive peptides was observed not only with Ac2-L-Lys-D-Ala (Fig. 2D) but also with the complete disaccharide tetrapeptide-(iAsn) peptidoglycan unit and with other donors containing a tetrapeptide ending in D-Ala. In contrast, no product was detected with Ac2-L-Lys-D-Ala-D-Ala and compounds containing a pentapeptide ending in D-Ala-D-Ala. Thus, Ldtfm catalyzes peptidoglycan cross-linking exclusively with tetrapeptide-containing donors that are formed in vivo by the β-lactam-insensitive D,D-carboxypeptidase according to the pathway depicted in Fig. 1B. Strikingly, the specificity of Ldtfm for a tetrapeptide donor ending in L-Lys3-D-Ala4 accounts for the lack of inhibition by β-lactams (Fig. 2E).
because the drugs are structural analogues of the d-Ala^3–d-Ala^5 extremity of the pentapeptide stem of peptidoglycan precursors. Cross-linking of Peptidoglycan Subunits in Vitro—Because Ldt_{fm} had all the characteristics expected for a peptidoglycan cross-linking enzyme, we investigated the formation of L-Lys^3–d-iAsn–L-Lys^3 cross-links with substrates closely mimicking the natural peptidoglycan precursors. Such substrates were prepared from the peptidoglycan of *E. gallinarum*, as it contains large amounts of uncrossed-linked monomers containing a tetrapeptide iAsn stem (19). LD-Transpeptidation was assayed with a reconstituted pool of three muropeptides to simultaneously test six combinations of donors and acceptors (Fig. 3A). Mass spectrometric analysis of the reaction products revealed formation of dimers with two types of donors (tetrapeptide and tetrapeptide-iAsn) and two types of acceptors (tetrapeptide-iAsn and tripeptide-iAsn) in the four possible combinations. The muropeptide containing an unsubstituted tetrapeptide stem was not used as an acceptor, indicating that the side-chain iAsn is essential. Accordingly, direct Lys^3–Lys^3 cross-links were not detected in the peptidoglycan of *E. faecium* M512 (12). To confirm the structure of the dimers obtained *in vitro*, the reaction was scaled up and treated with ammonium hydroxide to cleave the ether link internal to MurNAc. This treatment produced lactoyl peptides, which are more amenable to sequencing by tandem mass spectrometry than disaccharide peptides (21). This treatment was also found to convert D-iAsn into D-isoaspartyl. The fragmentation patterns (Fig. 3, B and C) demonstrated the *in vitro* formation of L-Lys^3–d-iAsn–L-Lys^3 cross-links by Ldt_{fm}. Of note, dimer formation has not been obtained in the case of purified DD-transpeptidase (PBPs), except in very special cases involving highly reactive artificial substrates (e.g. thioester) or atypical enzymes (e.g. the soluble R61 DD-peptidase from *Streptomyces spp.*) (see Ref. 24 for a recent discussion). Thus, Ldt_{fm} differs from the PBPs in its capacity to function in a soluble acellular system, a feature that could be exploited to design screens for the identification of cross-linking inhibitors.

Physiological Function of Ldt_{fm}—Similar Ldt_{fm} activity is present in crude extracts from the ampicillin-resistant *E. faecium* mutant M512 and from the susceptible parental strain D344S (13). Accordingly, Western blot analysis of crude extracts from *E. faecium* D344S and M512 revealed similar amounts of the same form of Ldt_{fm} corresponding to the full-length protein (data not shown). The identification of the corresponding gene, *ldt_{fm}*, allowed us to confirm that its sequence was identical in both strains and in the *E. faecium* genome database. These observations indicate that activation of the LD-transpeptidation pathway (Fig. 1B) does not involve modification of the activity of Ldt_{fm} per se but that of the supply of the appropriate tetrapeptide donor substrate for the cross-linking reaction. The physiological role of the LD-transpeptidase in β-lactam-susceptible *E. faecium* is unknown. Previous analyses of the peptidoglycan structure in *E. coli* revealed that a small proportion of the cross-links is generated by LD-transpeptidation during the exponential phase of growth (~5.8%) (25). An increase of their abundance during the stationary phase (~11.3%) was attributed to a short supply of peptidoglycan subunits containing the pentapeptide required for DD-transpeptidation (25). Because the mature peptidoglycan of *E. faecium* contains virtually no pentapeptide stems (12), we propose that Ldt_{fm} may have a role in the maintenance of peptidoglycan structure because the enzyme can catalyze new cross-links without *de novo* incorporation of pentapeptide-containing subunits.

Distribution of Ldt_{fm}—Sequence comparisons indicated that Ldt_{fm} is the first representative of a novel family of proteins that is sporadically distributed among taxonomically distant bacteria. Close homologues (Fig. 4) were detected in pathogenic Gram-positive bacteria including *Bacillus anthracis* and *Enterococcus faecalis* but not in *S. aureus* and *Streptococcus pneumoniae*. Sequence similarity restricted to the C terminus of Ldt_{fm} was also detected in proteins of unknown functions from other Gram-negative and Gram-positive bacteria, but the architecture and the domain composition of the proteins were different. This domain is related to 341 sequences from eu-bacteria appearing in the Protein Families Data Base of Alignments under the pfam accession number PF03734. Members of the family appear nearly ubiquitous, but none of them has been functionally characterized, and their putative involvement in β-lactam resistance has not been evaluated. Highly conserved residues of the C-terminal domain included Ser and Cys, present at positions 439 and 442 of Ldt_{fm} (Fig. 4) as potential catalytic residues. Site-directed mutagenesis of Ldt_{fm} led to an inactive protein for the C442A substitution. The mutant protein with the S439A substitution retained 2% of the activity of the wild-type enzyme. These results suggest that Cys~442~ could be the catalytic residue of Ldt_{fm}. In contrast, the PBPs possess an essential active site serine, which is acylated by their substrate and β-lactams.

In conclusion, Ldt_{fm} is the first characterized representative of a novel type of peptidoglycan transpeptidase. The wide distribution of Ldt_{fm} homologues indicates that β-lactam resistance by the LD-transpeptidase bypass mechanism can potentially emerge in various pathogenic bacteria. The specificity of the Ldt_{fm} for tetrapeptide substrates implies the lack of the β-lactam ring-associated activity of the most broadly used class of antimicrobial agents. This also implies that strategies applied previously to defeat other β-lactam resistance mechanisms, in particular by modification or replacement of the structures attached to the β-lactam ring, cannot be transposed to the development of LD-transpeptidase inhibitors.

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**β-Lactam-resistant Cell Wall Cross-linking**