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Identification of the L,D-Transpeptidases Responsible for Attachment of the Braun Lipoprotein to *Escherichia coli* Peptidoglycan▽

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The L,D-transpeptidase Ldt_{fm} catalyzes peptidoglycan cross-linking in β-lactam-resistant mutant strains of *Enterococcus faecium*. Here, we show that in *Escherichia coli* Ldt_{fm} homologues are responsible for the attachment of the Braun lipoprotein to murein, indicating that evolutionarily related domains have been tailored to use muropeptides or proteins as acyl acceptors in the L,D-transpeptidation reaction.

The peptidoglycan, which surrounds the bacterial cell, is polymerized from disaccharide-peptide subunits via the formation of glycoside and peptide bonds. Variations in the structure of mature peptidoglycan involve mainly the sequence of the stem peptide and its mode of cross-linking (15). In *Escherichia coli*, cross-linking of L-Ala[1]-D-Glu[2]-*meso*DAP[3]-D-Ala[4]-D-Ala[5] (where DAP is diaminopimelic acid) stem peptides occurs predominantly between the α-carboxyl of D-Ala[4] of one subunit and the ϵ-amine of *meso*DAP[3] of another subunit (4-3 cross-links). Cross-links involving two *meso*DAP[3] units (3-3 cross-links) are less abundant, representing 3 and 10% of the total muropeptide content in the exponential and stationary phases of growth, respectively (14). The 4-3 cross-links are formed by the D,D-transpeptidase activity of high-molecular-weight penicillin-binding proteins, while the enzymes responsible for the formation of 3-3 cross-links remain unknown in gram-negative bacteria. In gram-positive bacteria, an L,D-transpeptidase (Ldt_{fm}) has recently been shown to catalyze the formation of 3-3 cross-links in a β-lactam-resistant mutant strain of *Enterococcus faecium* selected in vitro (11) (Fig. 1A). This enzyme confers resistance by bypassing the β-lactam-sensitive D,D-transpeptidase activity of penicillin-binding proteins (12). The catalytic domain of Ldt_{fm} is the first functionally characterized member of a conserved family of proteins designated ErfK-YcfS-YhnG or pfam 03734 in databases. Additional members of this family from *Enterococcus faecalis* and *Bacillus subtilis* were also recently shown to catalyze peptidoglycan cross-linking in vitro (10). In this study, we have investigated by multiple chromosomal deletions and genetic complementation the role of four Ldt_{fm} homologues from *E. coli*.

Four genes, named *erfK*, *ycfS*, *ynhG*, and *ybiS*, encoding proteins which contain a domain related to the catalytic domain of Ldt_{fm} are present in the *E. coli* genome (Fig. 2). Strains carrying single and multiple deletions of the four genes were constructed from strain BW25113 by the procedure described by Datsenko and Wanner (7). The peptidoglycan of the parental strain and the quadruple-mutant (BW25113Δ4) strain was extracted (8) from stationary phase cultures, performed in brain heart infusion (BHI) broth at 37°C. Carbohydrates and proteins were digested with α-amylase and a mixture of proteases (Pronase; Roche), respectively (8). The purified peptidoglycan was digested with muramidases, and *N*-acetylmuramic acid (MurNAc) was reduced to muramitol by using sodium borohydride (2). The resulting muropeptides were separated by reverse-phase high-performance liquid chromatography (rp-HPLC) on a C_{18} column and analyzed by mass spectrometry (2). The relative abundance of muropeptides was estimated by the integration of the different peaks of the rp-HPLC profile. The dimer with a monoisotopic mass of 1,722.7, containing a *meso*DAP-*meso*DAP cross-link generated by L,D-transpeptidation, was detected in both strains (Fig. 3, peak 5), indicating that none of the four Ldt_{fm} homologues was required for the formation of 3-3 cross-links. The quadruple deletion suppressed the formation of muropeptides eluting in peaks 8, 13, and 14 in the wild-type elution profile (Fig. 3). These muropeptides differed from the monomer eluting in

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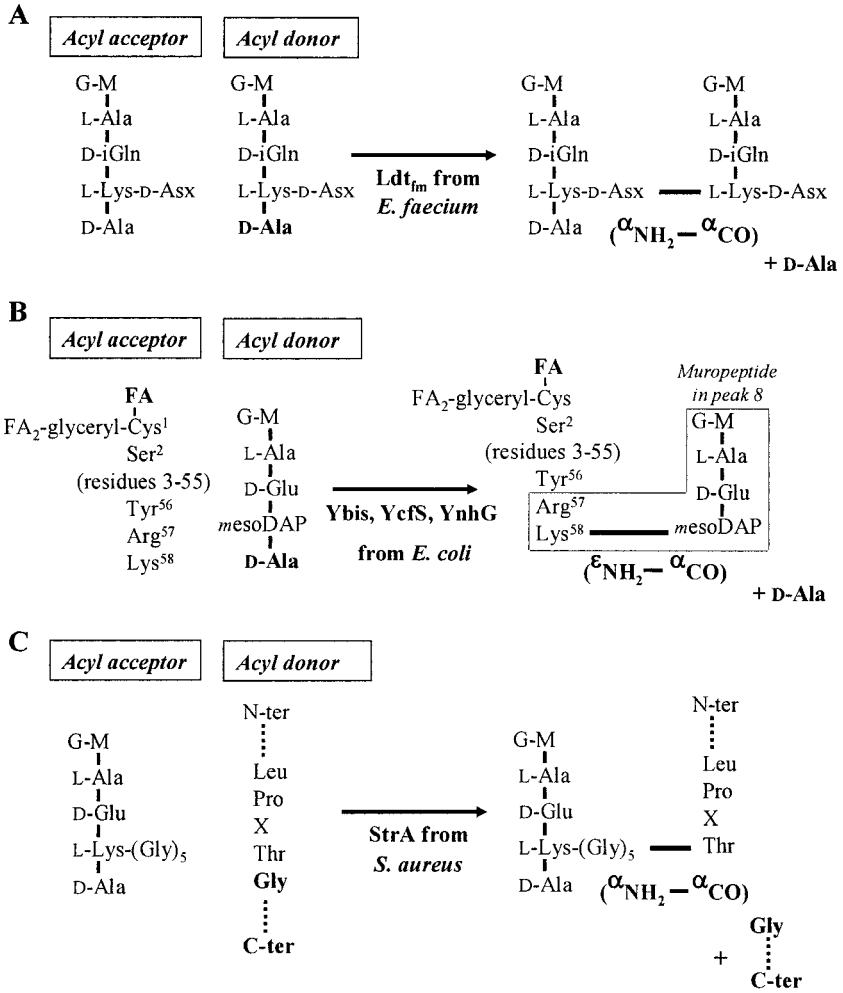


FIG. 1. Transpeptidation reactions catalyzed by Ldt$_{fm}$ from *E. faecium*, by YbisS from *E. coli*, and by the sortases from *Staphylococcus aureus*. (A) Ldt$_{fm}$ from *E. faecium* catalyzes the formation of 3-3 cross-links between two peptidoglycan subunits. The third position of the stem peptide consists of a D-Asp or a D-Asn residue (D-Asx) linked to the ε-amine of L-Lys via the β-carboxyl. The 3-3 cross-link connects the α-carboxyl of L-Lys in the donor to the α amine of D-Asx in the acceptor. (B) Anchoring of the Braun lipoprotein to the peptidoglycan in *E. coli*. The serine residue at position 2 (Ser^2) of the mature lipoprotein is the critical residue of a sorting signal for addressing the protein to the outer membrane (17). Attachment of three fatty acids (FA) to the N-terminal glyceryl-cysteine residue of the mature protein is responsible for its insertion into the outer membrane (6). The peptide bond formed by the YbiS L,D-transpeptidase links the α-carboxyl of *meso*DAP^3 of a disaccharide-peptide to the side chain amine of the C-terminal residue of the Braun lipoprotein (Lys^{58}). Muropeptide in peak 8 (boxed area) consists of a disaccharide-tripeptide substituted by the C-terminal dipeptide of the Braun lipoprotein (Lys^{58}-Arg^{57}) following cleavage of the Tyr^{56}-Arg^{57} peptide bond by pronase during peptidoglycan preparation. (C) Sortases catalyze the anchoring of proteins to the peptidoglycan of gram-positive bacteria. The StrA sortase from *S. aureus* cleaves the Thr-Gly peptide bond of the sorting signal (consensus sequence Leu-Pro-X-Thr-Gly [where X is any amino acid]) and links the α-carboxyl of Thr to the side chain amine at the third position of an acceptor disaccharide peptide (in which L-Lys is substituted by a pentaglycine in this bacterium). Residues flanking the sorting signal are represented by dotted lines. G-M, GlcNAc-MurNAc; D-iGln, D-isoglutamine.

peak 2 and from that of the dimers eluting in peaks 5 and 9 by a mass of 284.2, corresponding to the mass of the dipeptide Lys-Arg. Tandem mass spectrometry analysis of the monomer in peak 8 showed that Lys-Arg was linked via the ε-amine of Lys to the α-carboxyl of *meso*DAP^3 of a disaccharide-tripeptide (Fig. 1B, boxed area). Thus, this muropeptide contains a C-terminal dipeptide fragment of the Braun lipoprotein, known to be covalently linked to the peptidoglycan, as shown in Fig. 1B (5). These data indicate that the enzymes responsible for anchoring the major outer-membrane lipoprotein to the peptidoglycan of *E. coli* are related to the L,D-transpeptidases that catalyze the formation of 3-3 cross-links in gram-positive bacteria. In addition, deletion of the four genes uncovered an additional unknown L,D-transpeptidase, since the 3-3 cross-links persisted in the quadruple mutant.

Transcomplementation was performed to determine which of the four genes could restore attachment of the Braun lipoprotein to the peptidoglycan. Genes *erfK*, *ycfS*, *ynhG*, and *ybiS* were independently cloned into the pTrc99a expression vector (1), and the recombinant plasmids were introduced into *E. coli* BW25113Δ4. The transformants were grown in BHI broth containing ampicillin (150 μg/ml), and expression of the cloned genes was induced with isopropyl-β-D-thiogalactopyranoside (0.01 mM) at an optical density at 600 nm of 0.4.

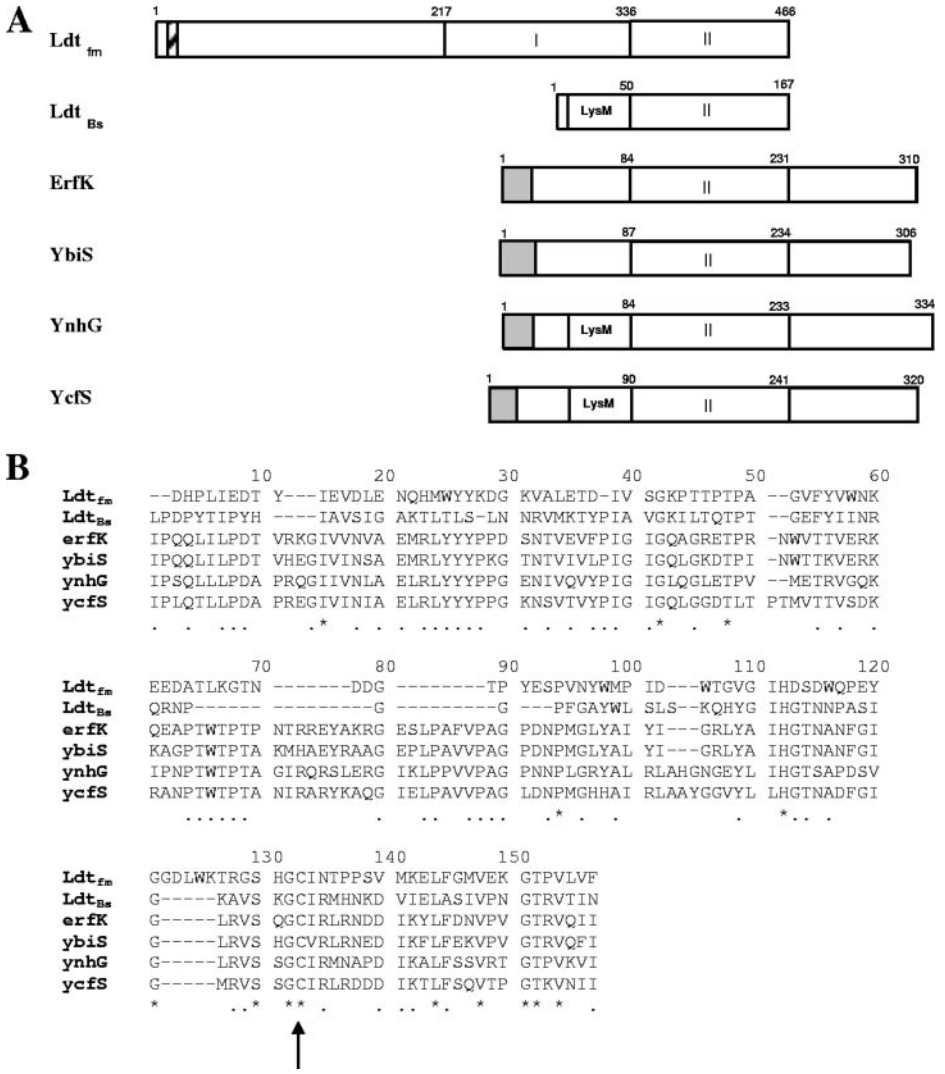
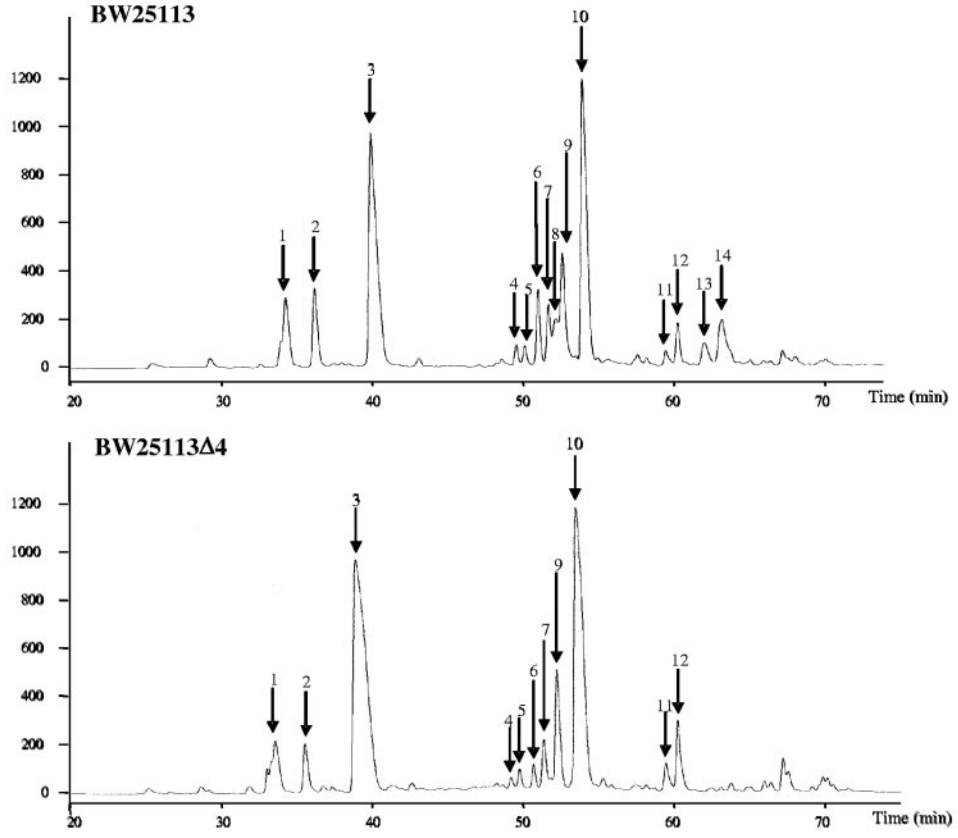


FIG. 2. Structure of Ldt_{fm} and related proteins. (A) Domain composition of L,D-transpeptidases from *E. faecium* (Ldt_{fm}) and *B. subtilis* (Ldt_{Bs}) and of the four homologues from *E. coli* (ErfK, YcfS, YnhG, and YbiS). The hatched box (upper left) represents the putative membrane anchor of Ldt_{fm} (11). The boxes labeled I and II represent structural domains of Ldt_{fm} (3). Homologues of the catalytic domain of Ldt_{fm} are present in the other proteins (also labeled as II). LysM designates a putative peptidoglycan-binding module. The four proteins from *E. coli* contain a putative peptide signal (gray boxes). Numbers indicate amino acid positions. (B) Sequence alignment of the putative catalytic domains (domain II). Amino acids conserved in the six proteins or only in the four proteins of *E. coli* are indicated by stars and dots, respectively. The catalytic Cys residue of Ldt_{fm} is indicated by an arrow.

Following further incubation at 37°C, peptidoglycan was extracted from stationary phase cultures and analyzed as described above for BW25113Δ4. Expression of three of the four genes, *erfK*, *ycfS*, and *ybiS*, restored the covalent anchoring of the lipoprotein to the peptidoglycan (data not shown). Thus, complementation analysis revealed that ErfK, YcfS, and YbiS can independently catalyze the covalent anchoring of the Braun lipoprotein to the peptidoglycan. However, this function appears to be performed mainly by YbiS in the parental strain since deletion of the *ybiS* gene alone almost completely suppressed peak 8, whereas deletion of *erfK*, *ycfS*, and *ynhG* alone or in combination had no effect on this peak (data not shown).

The muropeptide elution profiles of the various strains were also analyzed to determine whether additional functions could be associated with the Ldt_{fm} homologues. Expression of *ynhG* resulted in an increase of peaks 4 and 5, which correspond to muropeptides containing a 3-3 cross-link (from 0.4 to 7.4% for peak 4 and from 0.8 to 8.8% for peak 5). These results suggest that YnhG can contribute to L,D-transpeptidation of peptidoglycan subunits together with the unknown L,D-transpeptidase uncovered by deletion of the four ldt_{fm} homologues (see above). Because of the existence of this additional enzyme, however, it is not possible to exclude the possibility that the effect of *ynhG* expression on the abundance of 3-3 cross-links is only indirect. For example, the formation of tripeptide from pentapeptide by YnhG, as recently shown for the L,D-transpeptidase from *E. faecalis* (10), may modify the relative abundance of the substrates for the D,D-transpeptidation and L,D-transpeptidation reactions.

A



B

Peak	Oligomer	Muropeptide (cross-link)	Monoisotopic mass		
			Calculated	Observed for BW25113	Observed for BW25113Δ4
1	monomer	GM^R-Di	698.29	698.29	698.29
1	monomer	GM^R-Tri	870.37	870.38	870.38
2	monomer	GM^R-Tri -Gly	927.39	927.37	927.37
3	monomer	GM^R-Tetra	941.41	941.38	941.38
4	dimer	GM^R-Tri-Gly / GM^R-Tri (3-3)	1,779.75	1,779.80	1,779.80
5	dimer	GM^R-Tri / GM^R-Tri (3-3)	1,722.73	1,722.78	1,722.78
6	dimer	GM^R-Tri -Gly / GM^R-Tetra (3-3 or 4-3)	1,850.79	1,850.74	1,850.74
7	dimer	GM^R-Tetra / Tetra (4-3)	1,384.59	1,384.64	1,384.63
8	monomer	GM^R-Tri -L-Lys-L-Arg	1,154.57	1,154.57	ND
9	dimer	GM^R-Tetra / GM^R-Tri (3-3 or 4-3)	1,793.77	1,793.86	1,793.79
10	dimer	GM^R-Tetra / GM^R-Tetra (4-3)	1,864.80	1,864.74	1,864.85
11	trimer	GM^R-Tetra / GM^R-Tetra / GM^R-Tetra (4-3)	2,788.20	2,788.13	2,788.26
12	monomer	GM^A-Tetra	921.38	921.36	921.36
13	dimer	GM^R-Tri / GM^R-Tri -L-Lys-L-Arg (3-3)	2,006.93	2,006.98	ND
14	dimer	GM^R-Tetra / GM^R-Tri -L-Lys-L-Arg (4-3)	2,077.96	2,078.02	ND

FIG. 3. Peptidoglycan composition of the parental strain and of the quadruple mutant BW25113Δ4 obtained by deletion of genes *erfK*, *ycfS*, *ynhG*, and *ybiS*. (A) rp-HPLC profiles of muropeptides obtained by digestion of the peptidoglycan by muramidases. (B) Identification of muropeptides in the main peaks by mass spectrometry. GM^R, *N*-acetyl-glucosamine linked to reduced *N*-acetyl-muramic acid; GM^A, *N*-acetyl-glucosamine linked to anhydro-*N*-acetyl-muramic acid; Di, dipeptide L-Ala-D-Glu; Tri, tripeptide L-Ala-D-Glu-*meso*DAP; Tetra, tetrapeptide L-Ala-D-Glu-*meso*DAP-D-Ala. The type of cross-link (3-3 or 4-3) is indicated in parenthesis for dimers. ND, Not detected.

In conclusion, we have shown that the L,D-transpeptidases for peptidoglycan cross-linking in gram-positive bacteria and for attachment of the Braun lipoprotein to peptidoglycan in *E. coli* belong to the same protein family. The two reactions are expected to involve similar acyl donor but distinct acyl acceptor substrates (Fig. 1). The Ldt_{fm} of *E. faecium* cleaves the peptide bond between the third and fourth residues of a donor disaccharide-tetrapeptide and links the α-carboxyl of the third residue to the side chain amine at the third position of an acceptor disaccharide-tetrapeptide (Fig. 1A). In vivo, this reaction results in the formation of 3-3 cross-links between two stem peptides carried by adjacent glycan strands in the peptidoglycan layer. By analogy, ErfK, YcfS, and YbiS are expected to cleave the peptide bond between *meso*DAP^3 and D-Ala^4 in a donor disaccharide-tetrapeptide stem and to link the α-carboxyl of *meso*DAP^3 to the side chain amine of the L-Lys residue located at the C terminus of the Braun lipoprotein (Fig. 1B). In vivo, this reaction results in the anchoring of the C terminus of the Braun lipoprotein to the peptidoglycan layer. The N-terminal Cys residue of the mature Braun lipoprotein is also modified by the addition of fatty acid residues that insert into the outer membrane. Thus, the Braun lipoprotein, which folds in a stable trimeric structure (16), is thought to contribute to the integrity of the outer envelope structure by connecting the outer membrane to the peptidoglycan (4), although neither the loss of the protein (9) nor its anchoring to the peptidoglycan layer (this work) led to deleterious phenotypes (data not shown). In gram-positive bacteria, surface proteins are anchored to peptidoglycan by sortases which cleave a peptide bond within a sorting signal and link the carboxyl of the C-terminal residue to the side chain at the third position of a disaccharide-peptide (Fig. 1C). In this reaction, the protein acts as the carbonyl donor and the disaccharide-peptide acts as the acceptor. Sortases and L,D-transpeptidases of the Ldt_{fm} family are structurally unrelated, although both types of enzymes function with a catalytic Cys residue (11, 13).

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