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Identification of the L,D-Transpeptidases for Peptidoglycan Cross-Linking in *Escherichia coli*▽

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Three active-site cysteine L,D-transpeptidases can individually anchor the Braun lipoprotein to the *Escherichia coli* peptidoglycan. We show here that two additional enzymes of the same family form peptide bonds between the third residues of peptidoglycan stems, generating *meso*-DAP^3→*meso*-DAP^3 unusual cross-links. This activity partially replaces the D,D-transpeptidase activity of penicillin-binding proteins.

The peptidoglycan of *Escherichia coli* results from the polymerization of a disaccharide-peptide subunit containing a linear stem pentapeptide (Fig. 1). The final polymerization steps involve the formation of the glycan strands by glycosyltransferases and cross-linking of peptide stems by transpeptidases. The latter reaction is mainly catalyzed by D,D-transpeptidases that form D-Ala^4→*meso*-DAP^3 cross-links (4-3 cross-links; Fig. 1A). The D,D-transpeptidases belong to the penicillin-binding protein (PBP) family and are the targets of β-lactams. A small proportion of the cross-links are unlikely to be generated by PBPs since they involve two *meso*-DAP residues (*meso*-DAP^3→*meso*-DAP^3 or 3-3 cross-links) (5). The nature of the corresponding transpeptidase and its relationship to PBPs has remained speculative for several decades (6). Recently, L,D-transpeptidases that catalyze formation of 3-3 peptidoglycan cross-links have been identified in gram-positive bacteria (7, 9). In *Enterococcus faecium*, the enzyme can bypass the PBPs, resulting in high-level resistance to β-lactam antibiotics (11). The catalytic domain of the L,D-transpeptidases displays a new fold (1, 2) and contains an active-site Cys residue (9, 10) instead of Ser in PBPs (4).

In a recent attempt to identify the mode of synthesis of *meso*-DAP^3→*meso*-DAP^3 cross-links in *E. coli*, we deleted from the chromosome of strain BW25113 four genes encoding the proteins ErfK, YcfS, YbiS, and YnhG that display sequence similarity with the catalytic domain of L,D-transpeptidases from gram-positive bacteria (8). Unexpectedly, peptidoglycan analyses of the quadruple mutant, BW25113Δ4, and of its derivatives obtained by transcomplementation with each of the four genes have led to the identification of three L,D-transpeptidases that anchor the Braun lipoprotein to the peptidoglycan (ErfK, YcfS, and YbiS; Fig. 1B) (8). In contrast, the enzymes for synthesis of *meso*-DAP^3→*meso*-DAP^3 cross-links were not fully identified since overexpression of the fourth gene, *ynhG*, increased the abundance of 3-3 cross-links, whereas deletion of *ynhG* did not abolish their formation. In the present study, we identified a fifth L,D-transpeptidase, YcbB, and showed that this enzyme and YnhG are the only L,D-transpeptidases for synthesis of *meso*-DAP^3→*meso*-DAP^3 peptidoglycan cross-links in *E. coli* (Fig. 1C).

The *ycbB* gene was deleted from the chromosome of *E. coli* BW25113 using the procedure described by Datsenko and Wanner (3). Briefly, the linear PCR product used for gene replacement was obtained by amplification of the kanamycin resistance gene cassette of plasmid pKD4 with two primers (5′-TAAAATAACAGCCTGGCTATTCAGAGTATGATAAAAACAGGGGGCGTGTAGGCTGGAGCTGCTTC-3′ and 5′-AATCGCCCCCAATCATGCTAATTATTACGACAACTGATTTCCCCGCATATGAATATCCTCCTTAG-3′), which contained sequences flanking *ycbB* (underlined). Phage P1 was used to transduce the kanamycin cassette from the *ycbB* locus of *E. coli* BW25113 to that of the quadruple mutant *E. coli* BW25113Δ4 (8). The resulting mutant, BW25113Δ5, lacked *ycbB* in addition to *erfK*, *ycfS*, *ybiS*, and *ynhG*. Deletion of the five genes did not alter the growth characteristics in brain heart infusion broth (Difco). For transcomplementation analysis, the *ycbB* gene of *E. coli* BW25113 was amplified with the primers 5′-CGGAATTCTAAGGAGATACTA GATGTTGCTTAATATGATGTGTG-3′ and 5′-CCTCTAGATTACCTGATTAATTGTTCCGC-3, digested with EcoRI and XbaI (the sites are underlined), and cloned into the vector pTRC99a (Pharmacia) under the control of the p*lac* promoter. The resulting plasmid, pTRC99aΩ*ycbB*, was introduced into BW25113Δ5 using ampicillin (100 μg/ml) for selection. Expression of *ycbB* was induced with IPTG (isopropyl-β-D-thiogalactopyranoside; 0.02 mM) at an optical density of 0.8 at 600 nm in cultures performed in brain heart infusion broth at 37°C. The peptidoglycan was extracted at the stationary phase and digested with muramidases (8). The resulting muropeptides were separated by reversed-phase high-performance liquid chromatography (rp-HPLC) (Fig. 2A) and identified by mass spectrometry (Fig. 2B), as previously described (8).

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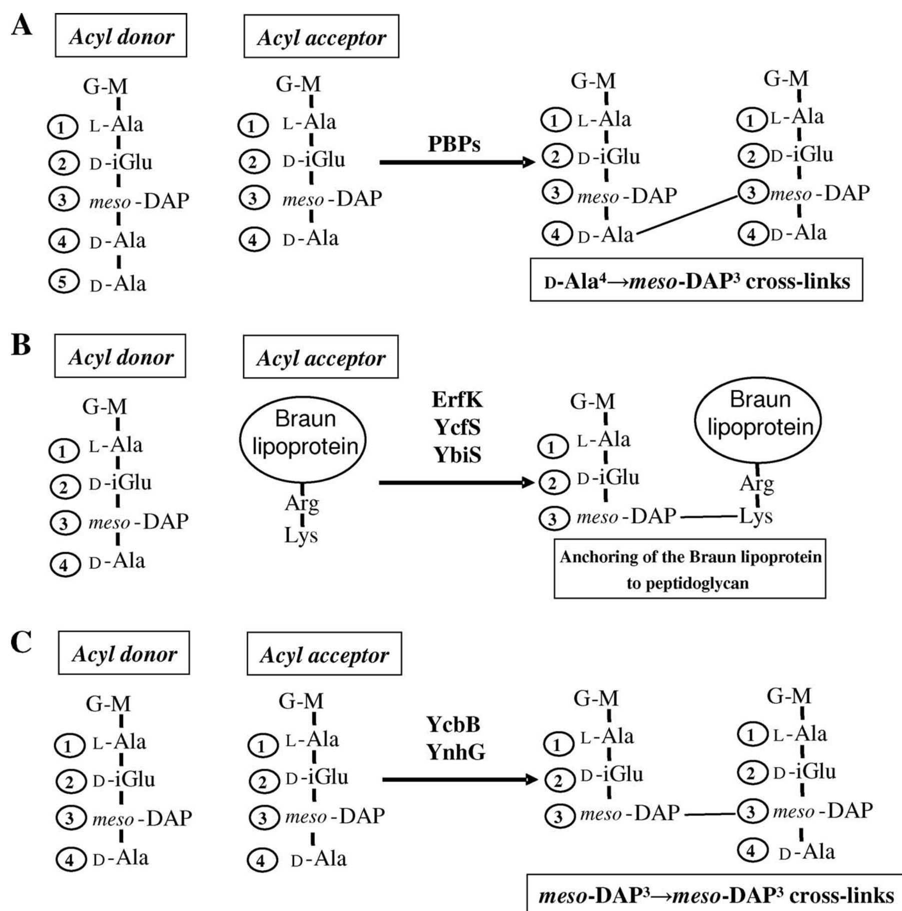
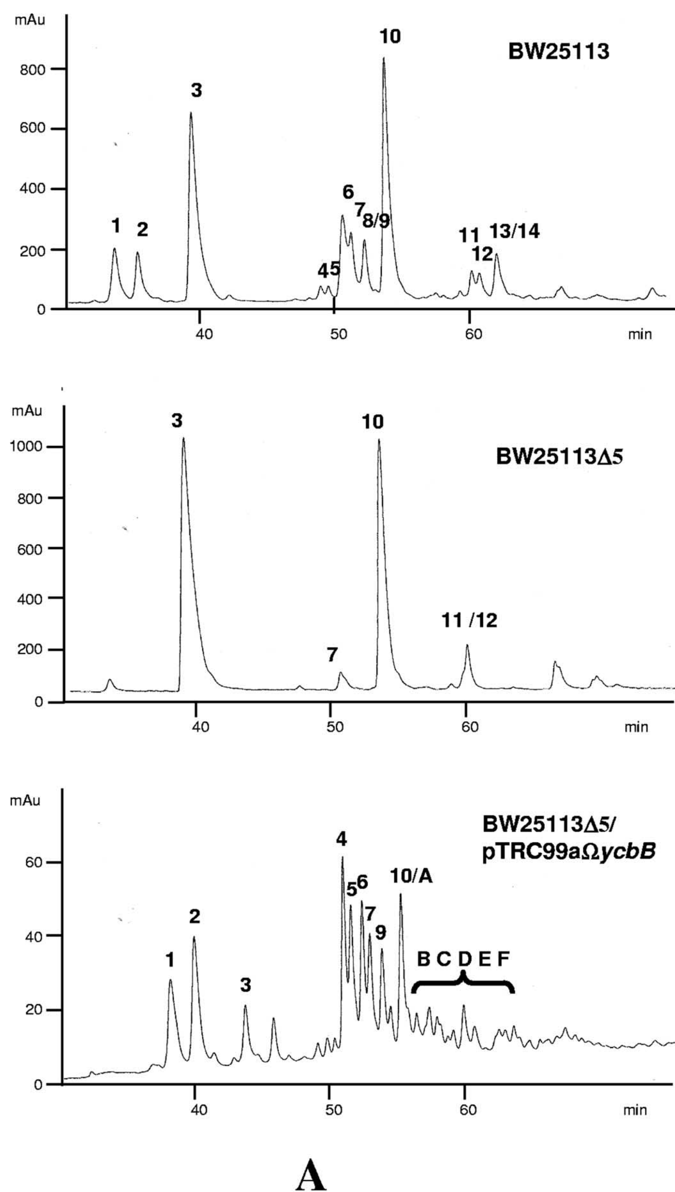


FIG. 1. Reaction catalyzed by the D,D-transpeptidases (PBPs) and the L,D-transpeptidases. (A) The disaccharide-peptide subunit is composed of β1-4-linked *N*-acetylglucosamine (G) and *N*-acetylmuramic acid (M) and a linear stem pentapeptide linked to the D-lactoyl group of *N*-acetylmuramic acid by an amide bond (5). The third position of the pentapeptide stem is occupied by a *meso*-diaminopymelyl residue (*meso*-DAP) linked to the γ-carboxyl of D-glutamic acid (L-Ala^1-D-iGlu^2-*meso*-DAP^3-D-Ala^4-D-Ala^5). PBPs cleave the D-Ala^4-D-Ala^5 peptide bond of a peptidoglycan donor stem and link the carbonyl of D-Ala^4 to the side chain amine of *meso*-DAP^3 in a peptidoglycan acceptor stem to form D-Ala^4→*meso*-DAP^3 cross-links. (B) The ErfK, YcfS, and YbiS L,D-transpeptidases anchor the Braun lipoprotein to peptidoglycan. These enzymes cleave the *meso*-DAP^3-D-Ala^4 peptide bond of a peptidoglycan donor stem and link the carbonyl of *meso*-DAP^3 to the side chain amine of the C-terminal Lys residue of the Braun lipoprotein. (C) The YcbB and YnhG L,D-transpeptidases generate the *meso*-DAP^3→*meso*-DAP^3 peptidoglycan cross-links. These enzymes cleave the *meso*-DAP^3-D-Ala^4 peptide bond of a peptidoglycan donor stem and link the carbonyl of *meso*-DAP^3 to the side chain amine of *meso*-DAP^3 in a peptidoglycan acceptor stem.

Deletion of the five genes encoding L,D-transpeptidases in *E. coli* BW25113Δ5 resulted in the disappearance of 9 out of the 14 muropeptides identified by rp-HPLC and mass spectrometry in the parental strain *E. coli* BW25113 (Fig. 2). The nine missing muropeptides included all of the muropeptides (8, 13, and 14) that contained a tripeptide stem substituted by a fragment of the Braun lipoprotein. This result was expected since BW25113Δ5 did not produce the L,D-transpeptidases previously shown to anchor the Braun lipoprotein to peptidoglycan (ErfK, YcfS, and YbiS; Fig. 1B) (8). The nine missing muropeptides also comprised all peptidoglycan dimers containing *meso*-DAP^3→*meso*-DAP^3 cross-links (muropeptides 4, 5, 9, and 13). This observation shows that *E. coli* does not produce any L,D-transpeptidase for formation of 3-3 cross-links in addition to YcbB (the present study) and YnhG (8). The quintuple deletion also led to the disappearance of muropeptides containing a free tripeptide stem (muropeptides 1, 5, and 9), indicating that the L,D-transpeptidases cleaved the *meso*-DAP^3-D-Ala^4 peptide bonds (L,D-carboxypeptidase activity). Finally, muropeptides containing a modified tetrapeptide stem ending in Gly instead of D-Ala^4 (muropeptides 2, 4, and 6) were absent, showing that Gly was used as an acyl acceptor resulting in the exchange of D-Ala^4 by Gly.

Expression of *ycbB* in BW25113Δ5 restored production of all missing muropeptides except those resulting from the anchoring of the Braun lipoprotein, revealing that the YcbB L,D-transpeptidase is sufficient for the formation of 3-3 cross-links, hydrolysis of D-Ala^4, and exchange of D-Ala^4 by Gly. In comparison to the parental strain, the abundance of these muropeptides was increased due to high-level expression of *ycbB* cloned into the expression vector pTRC99a. For example, the relative abundance of cross-links generated by L,D-transpeptidation was estimated to increase from 5 to 50% based on integration of the rp-HPLC peak areas (Fig. 2). Overexpression of *ycbB* also led to the formation of six additional muropeptides (A to F) due to an increase in the L,D-



Structure of the major muropeptides found in the rp-HPLC elution peaks

No	Structure	Cross-link
1	GM^{R}-Tri	
2	GM^{R}-Tri -Gly	
3	GM^{R}-Tetra	
4	GM^{R}-Tri-Gly / GM^{R}-Tri	(3-3)
5	GM^{R}-Tri / GM^{R}-Tri	(3-3)
6	GM^{R}-Tri -Gly / GM^{R}-Tetra	(4-3)
7	GM^{R}-Tetra / Tetra	(4-3)
8	GM^{R}-Tri -L-Lys-L-Arg	
9	GM^{R}-Tetra / GM^{R}-Tri	(3-3)
10	GM^{R}-Tetra / GM^{R}-Tetra	(4-3)
11	GM^{R}-Tetra / GM^{R}-Tetra / GM^{R}-Tetra	(4-3 and 4-3)
12	GM^{A}-Tetra	
13	GM^{R}-Tri / GM^{R}-Tri -L-Lys-L-Arg	(3-3)
14	GM^{R}-Tetra / GM^{R}Tri -L-Lys-L-Arg	(4-3)
A	GM^{A}-Tri	
B	GM^{A}-Tri-Gly	
C	GM^{R}-Tri-Gly / GM^{R}-Tri / GM^{R}-Tri	(3-3 and 3-3)
D	GM^{R}-Tetra / GM^{R}-Tri / GM^{R}-Tri	(3-3 and 3-3)
E	GM^{R}-Tri-Gly / GM^{R}-Tetra / GM^{R}-Tri	(4-3 and 3-3)
F	GM^{R}-Tetra / GM^{R}-Tetra / GM^{R}-Tri	(4-3 and 3-3)

A **B**

FIG. 2. Structure of the peptidoglycan of *E. coli* BW25113, BW25113Δ5, and BW25113Δ5/pTRC99aΩ*ycbB*. (A) rp-HPLC profiles of muropeptides obtained by digestion of the peptidoglycan by muramidases. Muropeptides 1 to 14 and A have been assigned to specific peaks detected by the absorbance at 210 nm. Muropeptides B to F could not be assigned to any specific absorbance peaks due to their low abundance; mAU, absorbance units (10^3). (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; Tri, tripeptide L-Ala-D-iGlu-*meso*-DAP; Tri-Gly, tetrapeptide L-Ala-D-iGlu-*meso*-DAP-Gly; Tetra, tetrapeptide L-Ala-D-iGlu-*meso*-DAP-D-Ala. The type of cross-links (3-3 or 4-3) is indicated in parentheses for dimers and trimers.

transpeptidase, L,D-carboxypeptidase, and exchange activities. For example, the additional muropeptides included a trimer containing two 3-3 cross-links (muropeptide C). This trimer was not detectable in the parental strain in which the substantial majority of the cross-links were generated by PBPs (4-3 cross-links).

In conclusion, we have shown that *E. coli* produces five L,D-transpeptidases with two distinct functions (Fig. 1). ErfK, YcfS, and YbiS anchor the Braun lipoprotein to the peptidoglycan, whereas YcbB and YnhG form the *meso*-DAP^{3}→*meso*-DAP^{3} peptidoglycan cross-links. In addition, all five L,D-transpeptidases appear to hydrolyze D-Ala^{4} and to exchange this residue with Gly since overexpression of the five L,D-transpeptidase genes individually led to the accumulation of tripeptide stems and of tetrapeptide stems ending in Gly.

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