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-DAP3→meso-DAP3 unusual cross-links. This activity partially replaces the L,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 and cross-linking of peptide stems by transpeptidases. In *Escherichia coli*, the enzyme can bypass the PBPs, resulting in high-level resistance to *β*-lactams. A small proportion of the cross-links are unlikely to be generated by PBPs since they involve two meso-DAP residues (meso-DAP3→meso-DAP3 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 linked to 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-DAP3→meso-DAP3 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-DAP3→meso-DAP3 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-DAP3→meso-DAP3 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′-TAAACTACAGCTTACATTGAGTA TGATAAAAACAGGGGCGTGTAAGGCTGGAGCTGC TTC3′ and 5′-AATTGCCCCAATCATGCTAATATTATG CAACAACGTATTTCCCCGATATGAATATCTCTCAGT AG3′), which contained sequences flanking ycbB (underlined). Phage P1 was used to transpose 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′-CGGAATTCAGGAGATACATA GTAGTGGCTTAATATGATGTGTG-3′ and 5′-CCTCTAGAT TTACCTCATAGATTTATATGCAACAACGTATTTCCCGATATGAATATCTCTCAGTAGT AG3′, which contained sequences flanking ycbB (underlined) (Fig. 2B), as previously described (8).
Deletion of the five genes encoding \( \text{L,D-transpeptidases} \) in \( \text{E. coli BW25113/H9004} \) resulted in the disappearance of 9 out of the 14 muropeptides identified by rp-HPLC and mass spectrometry in the parental strain \( \text{E. coli BW25113} \) (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 \( \text{BW25113/H9004} \) did not produce the \( \text{L,D-transpeptidases} \) 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 \( \text{meso-DAP}^3 \)-\( \text{meso-DAP}^3 \) cross-links (muropeptides 4, 5, 9, and 13). This observation shows that \( \text{E. coli} \) does not produce any \( \text{L,D-transpeptidase} \) for formation of \( \text{3-3 cross-links} \) in addition to \( \text{YcbB} \) (the present study) and \( \text{YnhG} \) (8). The quintuple deletion also led to the disappearance of muropeptides containing a free tripeptide stem (muropeptides 1, 5, and 9), indicating that the \( \text{L,D-transpeptidases} \) cleaved the \( \text{meso-DAP}^3 \)-\( \text{D-Ala}^4 \) peptide bonds (\( \text{L,D-carboxypeptidase activity} \)). Finally, muropeptides containing a modified tetrapeptide stem ending in Gly instead of \( \text{D-Ala}^4 \) (muropeptides 2, 4, and 6) were absent, showing that Gly was used as an acyl acceptor resulting in the exchange of \( \text{D-Ala}^4 \) by Gly.

Expression of \( \text{ycbB} \) in \( \text{BW25113/H9004} \) restored production of all missing muropeptides except those resulting from the anchoring of the Braun lipoprotein, revealing that the \( \text{YcbB L,D-transpeptidase} \) is sufficient for the formation of \( \text{3-3 cross-links} \), hydrolysis of \( \text{D-Ala}^4 \), and exchange of \( \text{D-Ala}^4 \) by Gly. In comparison to the parental strain, the abundance of these muropeptides was increased due to the high-level expression of \( \text{ycbB} \) cloned into the expression vector \( \text{pTRC99a} \). For example, the relative abundance of cross-links generated by \( \text{L,D-transpeptidation} \) was estimated to increase from 5 to 50% based on integration of the \( \text{rp-HPLC} \) peak areas (Fig. 2). Overexpression of \( \text{ycbB} \) also led to the formation of six additional muropeptides (A to F) due to an increase in the \( \text{L,D-} \)
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 \textit{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\(^3\) 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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