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

3
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16

17 **Running Title: *E. coli* lipoprotein attachment to peptidoglycan**

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23

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

29

30

31 The peptidoglycan, which surrounds the bacterial cell, is polymerized from disaccharide-
32 peptide subunits via formation of glycoside and peptide bonds. Variations in the structure of
33 mature peptidoglycan involve mainly the sequence of the stem peptide and its mode of cross-
34 linking (15). In *Escherichia coli*, cross-linking of L-Ala¹-D-Glu²-*meso*DAP³-D-Ala⁴-D-Ala⁵
35 (DAP: diaminopimelic acid) stem peptides occurs predominantly between the α -carboxyl of
36 D-Ala⁴ of one subunit and the ϵ -amine of *meso*DAP³ of another subunit (4-3 cross-links).
37 Cross-links involving two *meso*DAP³ (3-3 cross-links) are less abundant, representing 3 and
38 10 % of the total muropeptides content in the exponentially and stationary phases of growth,
39 respectively (14). The 4-3 cross-links are formed by the D,D-transpeptidase activity of high-
40 molecular weight penicillin-binding proteins (PBPs), while the enzymes responsible for the
41 formation of 3-3 cross-links remain unknown in gram-negative bacteria. In gram-positive
42 bacteria, an L,D-transpeptidase (Ldt_{fm}) has recently been shown to catalyze formation of 3-3
43 cross-links in a β -lactam-resistant mutant of *Enterococcus faecium* selected *in vitro* (11) (Fig.
44 1A). This enzyme confers resistance by by-passing the β -lactam-sensitive D,D-transpeptidase
45 activity of PBPs (12). The catalytic domain of Ldt_{fm} is the first functionally characterized
46 member of a conserved family of proteins designated ErfK-YcfS-YnhG or pfam 03734 in
47 databases. Additional members of this family from *Enterococcus faecalis* and *Bacillus subtilis*
48 were also recently shown to catalyze peptidoglycan cross-linking *in vitro* (10). In this study,
49 we have investigated by multiple chromosomal deletions and genetic complementation the
50 role of four Ldt_{fm} homologues from *E. coli*.

51 Four genes, named *erfK*, *ycfS*, *ynhG*, and *ybiS*, encoding proteins which contain a domain
52 related to the catalytic domain of Ldt_{fm} are present in the *E. coli* genome (Fig. 2). Single and
53 multiple deletions of the four genes were constructed in strain BW25113 by the procedure of
54 Datsenko and Wanner (7). The peptidoglycan of the parental strain and of the quadruple
55 mutant (BW25113 Δ 4) was extracted (8) from stationary phase cultures performed in Brain

56 Heart Infusion (BHI) broth at 37°C. Carbohydrates and proteins were digested with α -
57 amylase and a mixture of proteases (pronase from Roche), respectively (8). The purified
58 peptidoglycan was digested with muramidases and MurNAc was reduced to muramitol using
59 sodium borohydride (2). The resulting muropeptides were separated by rp-HPLC on a C18
60 column and analyzed by mass spectrometry (2). The relative abundance of muropeptides was
61 estimated by integration of the different peaks of the rp-HPLC profile. The dimer with a
62 monoisotopic mass of 1,722.7, containing a *meso*DAP-*meso*DAP cross-link generated by L,D-
63 transpeptidation, was detected in both strains (peak 5, Fig. 3) indicating that none of the four
64 *Ldt_{im}* homologues was required for formation of 3-3 cross-links. The quadruple deletion
65 suppressed formation of muropeptides eluting in peaks 8, 13, and 14 in the wild-type elution
66 profile (Fig. 3). These muropeptides differed from the monomer eluting in peak 2 and from
67 the dimers eluting in peaks 5 and 9 by a mass of 284.2 corresponding to the mass of the
68 dipeptide Lys-Arg. Tandem mass spectrometry analysis of the monomer in peak 8 showed
69 that Lys-Arg was linked via the ϵ -amine of Lys to the α -carboxyl of *meso*DAP³ of a
70 disaccharide-tripeptide (Boxed in Fig. 1B). Thus, this muropeptide contains a C-terminal
71 dipeptide fragment of the Braun lipoprotein, known to be covalently linked to the
72 peptidoglycan as depicted in Fig. 1B (5). These data indicate that the enzymes responsible for
73 anchoring the major outer membrane lipoprotein to the peptidoglycan of *E. coli* are related to
74 the L,D-transpeptidases that catalyze formation of 3-3 cross-links in gram-positive bacteria. In
75 addition, deletion of the four genes uncovered an additional unknown L,D-transpeptidase
76 since the 3-3 cross-links persisted in the quadruple mutant.

77 Transcomplementation was performed to determine which of the four genes could restore
78 attachment of the Braun lipoprotein to the peptidoglycan. The *erfK*, *ycfS*, *ynhG*, and *ybiS*
79 genes were independently cloned into the pTrc99a expression vector (1) and the recombinant
80 plasmids were introduced into *E. coli* BW25113 Δ 4. The transformants were grown in BHI

81 broth containing ampicillin (150 $\mu\text{g/ml}$) and expression of the cloned genes was induced with
82 isopropyl- β -D-thiogalactopyranoside (0.01 mM) at an OD of 0.4 at 600 nm. Following further
83 incubation at 37°C, peptidoglycan was extracted from stationary phase cultures and analyzed
84 as described above for BW25113 Δ 4. Expression of three out of the four genes, *erfK*, *ycfS*, and
85 *ybiS*, restored the covalent anchoring of the lipoprotein to the peptidoglycan (data not shown).
86 Thus, complementation analysis revealed that ErfK, YcfS, and YbiS can independently
87 catalyze the covalent anchoring of the Braun lipoprotein to the peptidoglycan. However, this
88 function appears to be mainly performed by YbiS in the parental strain since deletion of the *ybiS* gene
89 alone suppressed almost completely peak 8 whereas deletion of *erfK*, *ycfS*, and *ynhG*, alone or in
90 combination, had no effect on this peak (data not shown).

91 The muropeptide elution profiles of the various strains were also analyzed to determine
92 whether additional functions could be associated with the Ldt_{fm} homologues. Expression of
93 *ynhG* resulted in an increase of peaks 4 and 5, which correspond to muropeptides containing a
94 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
95 suggest that YnhG can contribute to L,D-transpeptidation of peptidoglycan subunits together
96 with the unknown L,D-transpeptidase uncovered by deletion of the four *ldt_{fm}* homologues (see
97 above). Because of the existence of this additional enzyme, it is however not possible to
98 exclude the possibility that the effect of *ynhG* expression on the abundance of 3-3 cross-links
99 is only indirect. For example, formation of tripeptide from pentapeptide by YnhG, as recently
100 shown for the L,D-transpeptidase from *E. faecalis* (10), may modify the relative abundance of
101 the substrates for the D,D-transpeptidation and L,D-transpeptidation reactions.

102 In conclusion, we have shown that the L,D-transpeptidases for peptidoglycan cross-
103 linking in gram-positive bacteria and for attachment of the Braun lipoprotein to peptidoglycan
104 in *E. coli* belong to the same protein family. The two reactions are expected to involve similar
105 acyl donor but distinct acyl acceptor substrates (Fig. 1). The L,D-transpeptidase of *E. faecium*

106 (Ldt_{fm}) cleaves the peptide bond between the third and fourth residue of a donor disaccharide-
107 tetrapeptide and links the α -carboxyl of the third residue to the side chain amine at the third
108 position of an acceptor disaccharide-tetrapeptide (Fig. 1A). *In vivo*, this reaction results in the
109 formation of 3-3 cross-links between two stem peptides carried by adjacent glycan strands in
110 the peptidoglycan layer. By analogy, ErfK, YcfS, and YbiS are expected to cleave the peptide
111 bond between *meso*DAP³ and D-Ala⁴ in a donor disaccharide-tetrapeptide stem and to link the
112 α -carboxyl of *meso*DAP³ to the side chain amine of the L-Lys residue located at the C-
113 terminus of the Braun lipoprotein (Fig. 1B). *In vivo*, this reaction results in the anchoring of
114 the C-terminus of the Braun lipoprotein to the peptidoglycan layer. The N-terminal Cys
115 residue of the mature Braun lipoprotein is also modified by the addition of fatty acid residues
116 that insert into the outer membrane. Thus, the Braun lipoprotein, which folds in a stable
117 trimeric structure (16), is thought to contribute to the integrity of the outer envelope structure
118 by connecting the outer membrane to the peptidoglycan (4), although neither the loss of the
119 protein (9) nor its anchoring to the peptidoglycan layer (this work) lead to deleterious
120 phenotypes (data not shown). In gram-positive bacteria surface proteins are anchored to
121 peptidoglycan by sortases which cleave a peptide bond within a sorting signal and link the
122 carboxyl of the C-terminal residue to the side chain at the third position of a disaccharide-
123 peptide (Fig. 1C). In this reaction, the protein acts as the carbonyl donor and the disaccharide-
124 peptide as the acceptor. Sortases and L,D-transpeptidases of the Ldt_{fm} family are structurally
125 unrelated although both types of enzymes function with a catalytic Cys residue (11, 13).

126

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130

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FIGURE LEGENDS

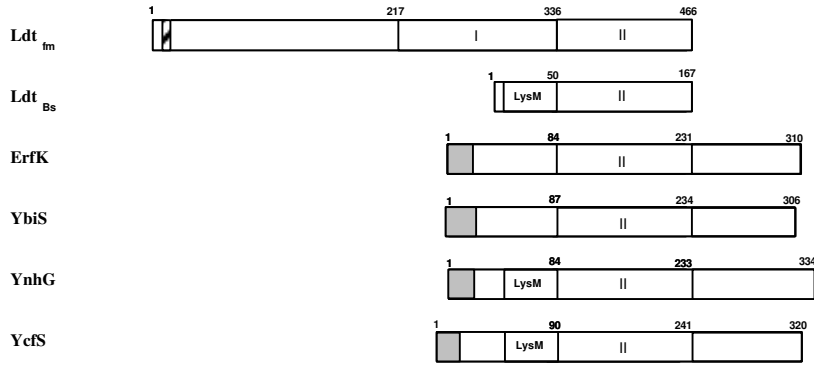
182
183
184 Figure 1. Transpeptidation reactions catalyzed by Ldt_{fm} from *E. faecium*, YbiS from *E. coli*,
185 and by the sortases from *Staphylococcus aureus*. (A) Ldt_{fm} from *E. faecium* catalyzes
186 formation of 3-3 cross-links between two peptidoglycan subunits. The third position of the
187 stem peptide consists of a D-Asp or D-Asn residue (D-Asx) linked to the ε-amine of L-Lys via
188 their β-carboxyl. The 3-3 cross-link connects the α-carboxyl of D-Ala in the donor to the α
189 amine of D-Asx in the acceptor. (B) Anchoring of the Braun lipoprotein to the peptidoglycan
190 in *E. coli*. The Ser residue at position 2 of the mature lipoprotein is the critical residue of a
191 sorting signal for addressing the protein to the outer membrane (17). Attachment of three
192 fatty-acids (FA) to the N-terminal glyceryl-cysteine residue of the mature protein is
193 responsible for its insertion into the outer membrane (6). The peptide bond formed by the
194 YbiS L,D-transpeptidase links the α-carboxyl of *meso*DAP³ of a disaccharide-peptide to the
195 side chain amine of the C-terminal residue of the Braun lipoprotein (Lys⁵⁸). Muropetide 8
196 (boxed) consists of a disaccharide-tripeptide substituted by the C-terminal dipeptide of the
197 Braun lipoprotein (Lys⁵⁸-Arg⁵⁷) following cleavage of the Tyr⁵⁶-Arg⁵⁷ peptide bond by
198 pronase during peptidoglycan preparation. (C) Sortases catalyze anchoring of proteins to the
199 peptidoglycan of gram-positive bacteria. The StrA sortase from *S. aureus* cleaves the Thr-Gly
200 peptide bond of the sorting signal (consensus sequence Leu-Pro-X-Thr-Gly; X, any amino
201 acid) and links the α-carboxyl of Thr to the side chain amine at the third position of an
202 acceptor disaccharide peptide (containing L-Lys substituted by a pentaglycine in this
203 bacterium). Residues flanking the sorting signal are representing by dot lines. G-M, GlcNAc-
204 MurNAc. D-iGln, D-isoglutamine.

205

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

216
217 Figure 3. Peptidoglycan composition of the parental strain and of the quadruple mutant
218 BW25113Δ4 obtained by deletion of the *erfK*, *ycfS*, *ynhG*, and *ybiS* genes. (A) rp-HPLC
219 profiles of muropeptides obtained by digestion of the peptidoglycan by muramidases. (B)
220 Identification of muropeptides in the main peaks by mass spectrometry. GM^R, *N*-acetyl-
221 glucosamine linked to reduced *N*-acetyl-muramic acid; GM^A, *N*-acetyl-glucosamine linked to
222 anhydro-*N*-acetyl-muramic acid; Di, dipeptide L-Ala-D-Glu; Tri, tripeptide L-Ala-D-iGlu-
223 *meso*DAP; Tetra, tetrapeptide L-Ala-D-iGlu-*meso*DAP-D-Ala. The type of cross links (3-3 or
224 4-3) is indicated in parenthesis for dimers. ND, Not detected.

A



B

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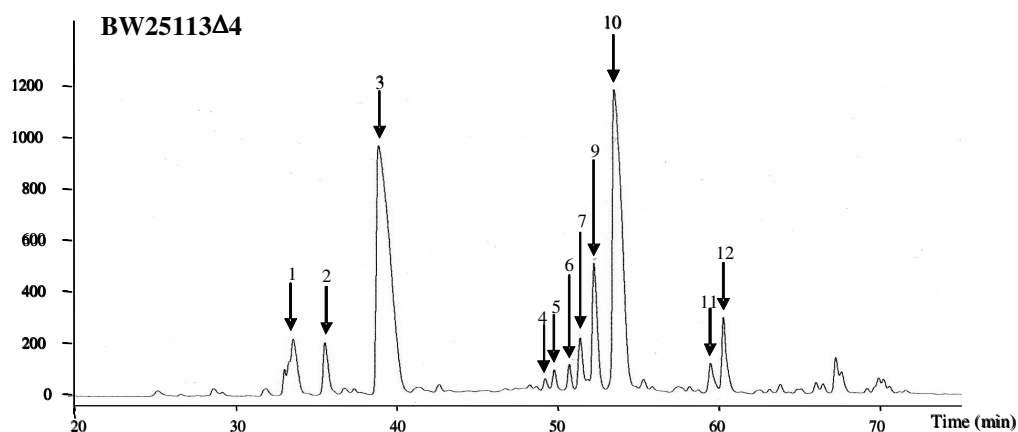
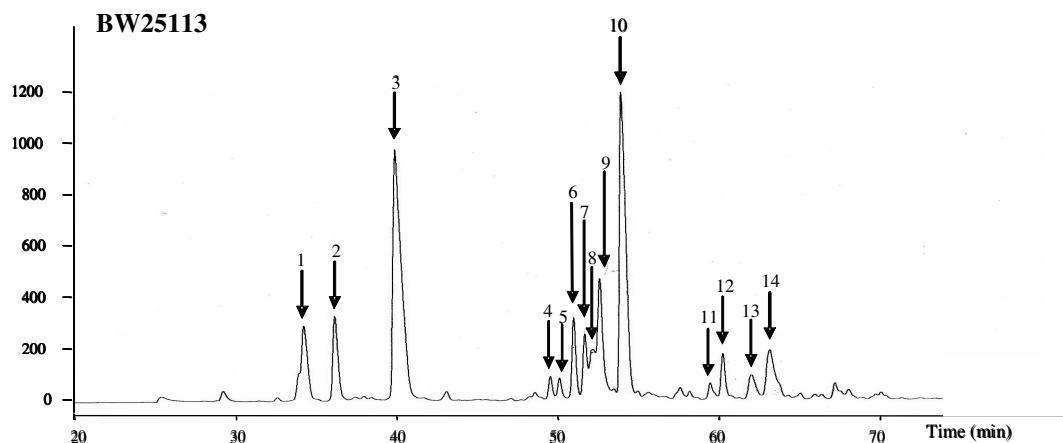
      10      20      30      40      50      60
Ldtfm  --DHPLIEDT Y---IEVDLE NQHMYYYKDG KVALETID-IV SGKPTTPTPA --GVFYVWNK
LdtBs  LPDPYTIPIYH ----IAVSIK AKTLTLS-LN NRVMKTYPIA VGKILTQTPT --GEFYIINR
erfK  IPQQLILPDT VRKGIVVNVA EMRLYYPPD SNTVEVFPPIG IGQAGRETPT -NWVTIVERK
ybiS  IPQQLILPDT VHEGIVINSA EMRLYYPKG TINTVIVLPIG IGQLGKDTPI -NWTTKVERK
ynhG  IPSQLLLPDA PRQGIIVNLA ELRLYYPPG ENIVQVYPIG IGLQGLETPV --METRVGQK
ycfS  IPLQTLPPDA PREGIVINIA ELRLYYPPG KNSVTVYPIG IGQLGGDILT PTMVTTVSDK
      . . . . . * . . . . . . . . . . * . . . . .

      70      80      90      100     110     120
Ldtfm  EEDATLKGIN -----DDG -----TP YESPVNYWMP ID---WTGVG IHDSDWQPEY
LdtBs  QRNP-----G -----G ---PFGAYWL SLS--KQHYG IHGTNNPASI
erfK  QEAPTWTPTP NTRREYAKRG ESLPAFVPAG PDNPMGLYAI YI---GRLYA IHGTNANFGI
ybiS  KAGPTWTPTA KMHAEYRAAG EPLPAVVPAG PDNPMGLYAL YI---GRLYA IHGTNANFGI
ynhG  IPNFTWTPTA GIRQRSLERG IKLPPVVPAG PNNPLGRYAL RLAHNGEYLL IHGTSAPDSV
ycfS  RANFTWTPTA NIRARYKAQG IELPAVVPAG LDNPMGHAI RLAAYGGVYL LHGTNADFGI
      . . . . . . . . . . . * . . . . . * . . . . .

      130     140     150
Ldtfm  GGDLWKTRGS HGCINTPPSV MKELFGMVEK GTPVLVF
LdtBs  G----KAVS KGCIRMHNKD VIELASIVPN GTRVTIN
erfK  G----LRVS QGCIRLRNDD IKYLFDNVPV GTRVQII
ybiS  G----LRVS HGCVRLRNEE IKFLFEKVPV GTRVQFI
ynhG  G----LRVS SGCIRMNAPD IKALFSSVRT GTPVKVI
ycfS  G----MRVS SGCIRLRDDD IKTLFSQVTP GTKVNII
      * . . . . * * . . . . * * * * .
    
```



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 |