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

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The Ldt_{fm} L,D-transpeptidase catalyzes peptidoglycan cross-linking in β-lactam-resistant mutants of *Enterococcus faecium*. Here, we show that in *Escherichia coli* Ldt_{fm} homologues are responsible for attachment of the Braun lipoprotein to murein indicating that evolutionary-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 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 (DAP: 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 (3-3 cross-links) are less abundant, representing 3 and 10 % of the total muropeptides content in the exponentially 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 (PBPs), 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 formation of 3-3 cross-links in a β-lactam-resistant mutant of *Enterococcus faecium* selected *in vitro* (11) (Fig. 1A). This enzyme confers resistance by by-passing the β-lactam-sensitive D,D-transpeptidase activity of PBPs (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). Single and multiple deletions of the four genes were constructed in strain BW25113 by the procedure of Datsenko and Wanner (7). The peptidoglycan of the parental strain and of the quadruple mutant (BW25113Δ4) 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 from Roche), respectively (8). The purified peptidoglycan was digested with muramidases and MurNAc was reduced to muramitol using sodium borohydride (2). The resulting muropeptides were separated by rp-HPLC on a C18 column and analyzed by mass spectrometry (2). The relative abundance of muropeptides was estimated by 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 (peak 5, Fig. 3) indicating that none of the four Ldt_{fm} homologues was required for formation of 3-3 cross-links. The quadruple deletion suppressed 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 peak 2 and from 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 (Boxed in Fig. 1B). Thus, this muropeptide contains a C-terminal dipeptide fragment of the Braun lipoprotein, known to be covalently linked to the peptidoglycan as depicted 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 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. The *erfK*, *ycfS, ynhG, and ybiS* genes 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 OD of 0.4 at 600 nm. Following further incubation at 37°C, peptidoglycan was extracted from stationary phase cultures and analyzed as described above for BW25113Δ4. Expression of three out 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 mainly performed by YbiS in the parental strain since deletion of the *ybiS* gene alone suppressed almost completely 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, it is however 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, 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.

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 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*DAP^3 and D-Ala^4 in a donor disaccharide-tetrapeptide stem and to link the
112 α-carboxyl of *meso*DAP^3 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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REFERENCES

1. **Amann, E., B. Ochs, and K. J. Abel.** 1988. Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69:**301-15.
2. **Arbeloa, A., J. E. Hugonnet, A. C. Sentilhes, N. Josseaume, L. Dubost, C. Monsempes, D. Blanot, J. P. Brouard, and M. Arthur.** 2004. Synthesis of mosaic peptidoglycan cross-bridges by hybrid peptidoglycan assembly pathways in gram-positive bacteria. J. Biol. Chem. **279:**41546-56.
3. **Biarrotte-Sorin, S., J. E. Hugonnet, V. Delfosse, J. L. Mainardi, L. Gutmann, M. Arthur, and C. Mayer.** 2006. Crystal structure of a novel beta-lactam-insensitive peptidoglycan transpeptidase. J. Mol. Biol. **359:**533-8.
4. **Braun, V.** 1975. Covalent lipoprotein from the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta. **415:**335-77.
5. **Braun, V., and K. Rehn.** 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall. The specific effect of trypsin on the membrane structure. Eur. J. Biochem. **10:**426-38.
6. **Choi, D. S., H. Yamada, T. Mizuno, and S. Mizushima.** 1986. Trimeric structure and localization of the major lipoprotein in the cell surface of *Escherichia coli*. J. Biol. Chem. **261:**8953-7.
7. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640-5.
8. **Glauner, B.** 1988. Separation and quantification of muropeptides with high-performance liquid chromatography. Anal. Biochem. **172:**451-64.

9. **Hirota, Y., H. Suzuki, Y. Nishimura, and S. Yasuda.** 1977. On the process of cellular division in *Escherichia coli*: a mutant of *E. coli* lacking a murein-lipoprotein. Proc. Natl. Acad. Sci. USA **74:**1417-20.

10. **Magnet, S., A. Arbeloa, J. L. Mainardi, J. E. Hugonnet, M. Fourgeaud, L. Dubost, A. Marie, V. Delfosse, C. Mayer, L. B. Rice, and M. Arthur.** 2007. Specificity of L,D-transpeptidases from Gram-positive bacteria producing different peptidoglycan chemotypes. J. Biol. Chem. (E-pub)

11. **Mainardi, J., Fourgeaud M, Hugonnet JE, Dubost L, Brouard JP, Ouazzani J, Rice LB, Gutmann L, Arthur M.** 2005. A novel peptidoglycan cross-linking enzyme for a beta-lactam-resistant transpeptidation pathway. J. Biol. Chem. **280:**38146-52.

12. **Mainardi, J. L., V. Morel, M. Fourgeaud, J. Cremniter, D. Blanot, R. Legrand, C. Frehel, M. Arthur, J. Van Heijenoort, and L. Gutmann.** 2002. Balance between two transpeptidation mechanisms determines the expression of beta-lactam resistance in *Enterococcus faecium*. J. Biol. Chem. **277:**35801-7.

13. **Marraffini, L. A., A. C. Dedent, and O. Schneewind.** 2006. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. Microbiol. Mol. Biol. Rev. **70:**192-221.

14. **Pisabarro, A. G., M. A. de Pedro, and D. Vazquez.** 1985. Structural modifications in the peptidoglycan of *Escherichia coli* associated with changes in the state of growth of the culture. J. Bacteriol. **161:**238-42.

15. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36:**407-77.

16. **Shu, W., J. Liu, H. Ji, and M. Lu.** 2000. Core structure of the outer membrane lipoprotein from *Escherichia coli* at 1.9 A resolution. J. Mol. Biol. **299:**1101-12.

17. **Yakushi, T., T. Tajima, S. Matsuyama, and H. Tokuda.** 1997. Lethality of the covalent linkage between mislocalized major outer membrane lipoprotein and the peptidoglycan of *Escherichia coli*. J. Bacteriol. **179:**2857-62.

182 **FIGURE LEGENDS**

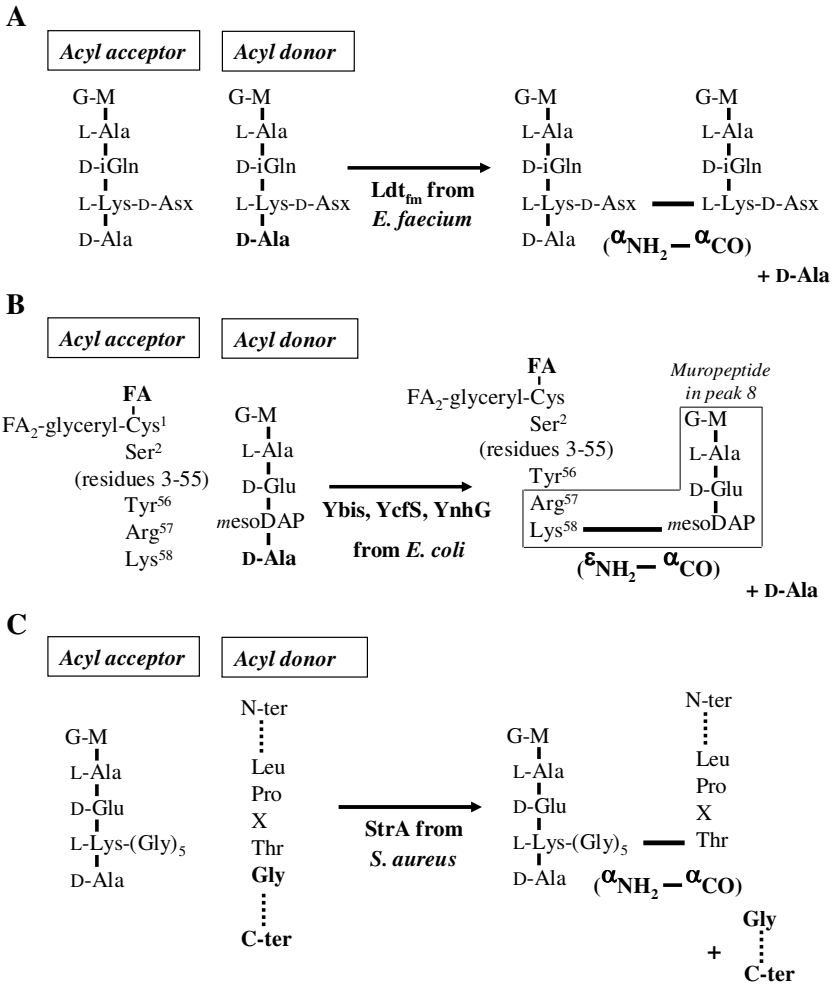
183

184 Figure 1. Transpeptidation reactions catalyzed by Ldt_{fm} from *E. faecium*, YbisS 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*DAP^3 of a disaccharide-peptide to the
195 side chain amine of the C-terminal residue of the Braun lipoprotein (Lys^{58}). Muropetide 8
196 (boxed) consists of a disaccharide-tripeptide substituted by the C-terminal dipeptide of the
197 Braun lipoprotein (Lys^{58}-Arg^{57}) following cleavage of the Tyr^{56}-Arg^{57} 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

Figure 2. Structure of Ldt_{fm} and related proteins. (A) Domain composition of L,D-transpeptidases from *E. faecium* (Ldt_{fm}), *B. subtilis* (Ldt_{Bs}), and of the four homologues from *E. coli* (ErfK, YcfS, YnhG, and YbiS). The hatched box represents the putative membrane anchor of Ldt_{fm} (11). The boxes labelled 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 (grey boxes). Numbers indicated 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.

Figure 3. Peptidoglycan composition of the parental strain and of the quadruple mutant BW25113Δ4 obtained by deletion of the *erfK*, *ycfS*, *ynhG,* and *ybiS* genes. (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-iGlu-*meso*DAP; Tetra, tetrapeptide L-Ala-D-iGlu-*meso*DAP-D-Ala. The type of cross links (3-3 or 4-3) is indicated in parenthesis for dimers. ND, Not detected.



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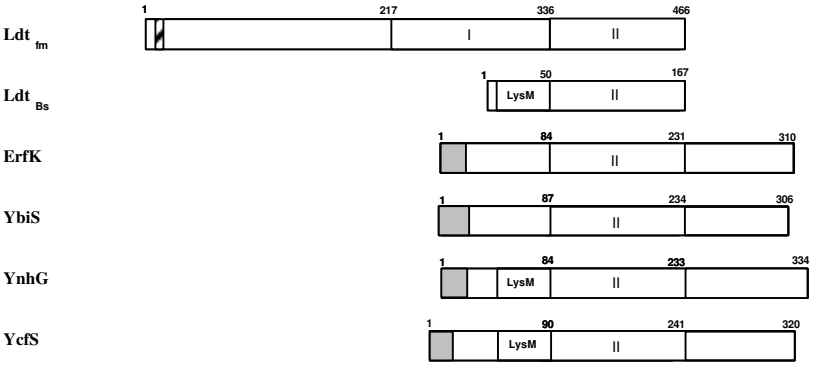

B

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                10         20         30         40         50         60
Ldtfm   --DHPLIEDT Y---IEVDLE NQHMWYYKDG KVALETD-IV SGKPTTPTPA --GVFYVWNK
LdtBs   LPDPYTIPYH ----IAVSIG AKTLTLS-LN NRVMKTYPIA VGKILTQTPT --GEFYIINR
erfK    IPQQLILPDT VRKGIVVNVA EMRLYYYPPD SNTVEVFPIG IGQAGRETPR -NWVTTVERK
ybiS    IPQQLILPDT VHEGIVINSA EMRLYYYPKG TNTVIVLPIG IGQLGKDTPI -NWTTKVERK
ynhG    IPSQLLLPDA PRQGIIVNLA ELRLYYYPPG ENIVQVYPIG IGLQGLETPV --METRVGQK
ycfS    IPLQTLLPDA PREGIVINIA ELRLYYYPPG KNSVTVYPIG IGQLGGDTLT PTMVTTVSDK
        . .  ...    .* . . . ......  . . . ...  .*  .  *      . .  .

                70         80         90        100        110        120
Ldtfm   EEDATLKGTN -------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    IPNPTWTPTA GIRQRSLERG IKLPPVVPAG PNNPLGRYAL RLAHGNGEYL IHGTSAPDSV
ycfS    RANPTWTPTA NIRARYKAQG IELPAVVPAG LDNPMGHHAI 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 HGCVRLRNED IKFLFEKVPV GTRVQFI
ynhG    G-----LRVS SGCIRMNAPD IKALFSSVRT GTPVKVI
ycfS    G-----MRVS SGCIRLRDDD IKTLFSQVTP GTKVNII
*       ..*  ** .    . .. *.  *   ** *  .
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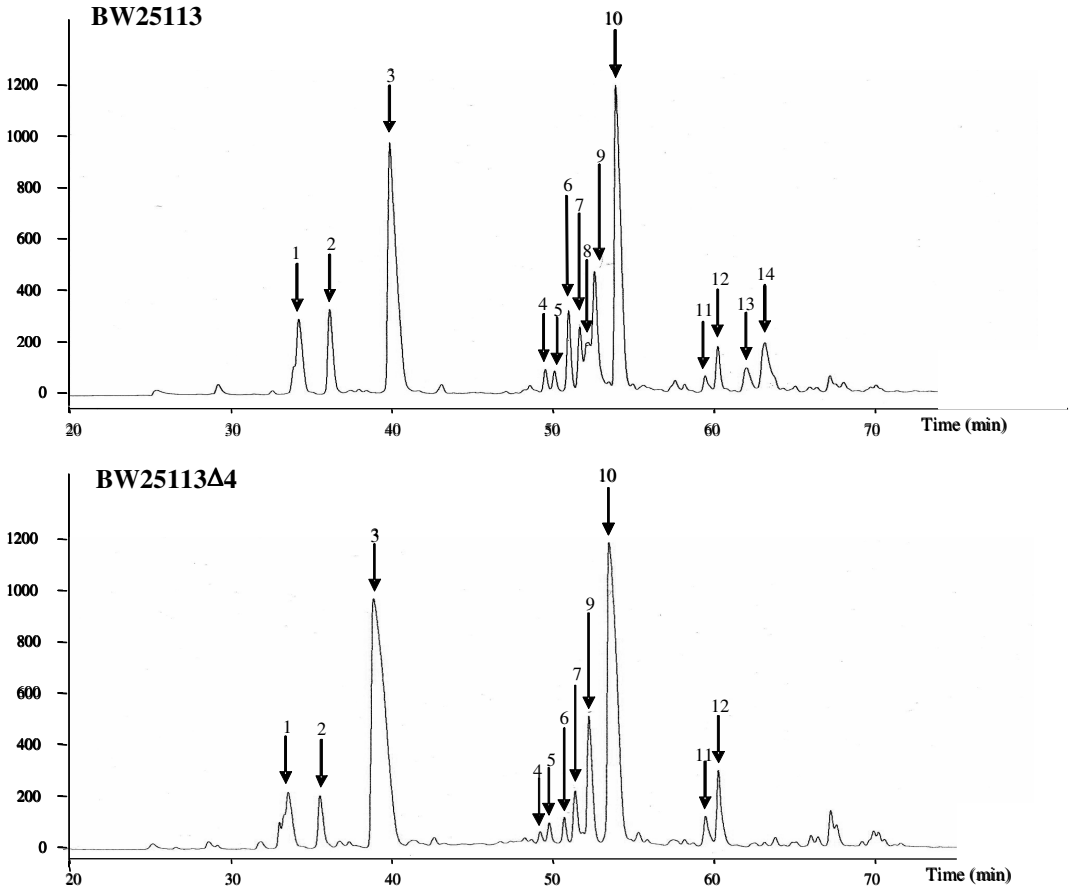
A



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