Supplemental Information:

Cleavage of Braun's lipoprotein Lpp from the bacterial peptidoglycan by a paralog of L,D-transpeptidases, LdtF

Raj Bahadur, Pavan Kumar Chodisetti, and Manjula Reddy*

CSIR-Centre for Cellular and Molecular Biology

Hyderabad India 500007

Supporting Materials and Methods

Plasmid constructions

For PCR amplifications, genomic DNA of MG1655 strain was used as a template unless otherwise indicated. Amplification of DNA for cloning purposes was done using Phusion DNA polymerase (NEB) and clones obtained were confirmed by sequence analysis.

pRB1. The *ldtF* gene along with its native ribosome binding site (RBS) was PCR amplified using forward and reverse primers 5'-GCTCTAGAAGGAATAAGCAGTATGCGTAAA-3' and 5'-CCCAAGCTTTTATTTTGCCTCGGGGAGCGTGT-3' respectively and the resulting amplified DNA fragment was cloned using XbaI and HindIII sites (underlined in the primer sequence) in a cloning vector, pTrc99a to obtain pRB1. The clones were confirmed by sequence analysis and shown to suppress the NA-sensitivity of Δ*mepS* mutant at 37°C with 250 μM IPTG.

pRB2 and pRB3. To create site directed variants of *ldtF* (H135A and C143A), a 3 step PCR was performed. For this procedure, two primers were synthesized that are complementary to each other with desired mutations at the center (in bold and underlined). In the first PCR step, N-terminal fragment of *ldtF* gene was amplified using a common forward primer and a reverse primer containing the desired mismatch. In the second PCR step, C-terminal fragment of *ldtF* gene was amplified using a forward primer containing the desired mismatch and a common reverse primer. Desired mismatches code for an alanine instead of H135, and C143 in the LdtF. Common forward primer (containing its native RBS and an XbaI site), and reverse primer (containing a HindIII site) are used for cloning these variants.

Common forward primer: 5'-GCTCTAGAAGGAATAAGCAGTATGCGTAAA-'3 and

Reverse primer: 5'-CCCAAGCTTTTATTTTGCCTCGGGGAGCGTGT-3'.

The forward and reverse primers with nucleotide substitution are:

Histidine to alanine change at codon 135-

5’-AAGGGAAATACCTGATGATC**GCT**GGCGATTGTGTTTCCATCGG-3’ and

5’-CCGATGGAAACACAATCGCC**AGC**GATCATCAGGTATTTCCCTT-3’

Cysteine to alanine change at codon 143-

5’-GCGATTGTGTTTCCATCGGC**GCT**TACGCAATGACCAATCAGGG-3’ and

5’- CCCTGATTGGTCATTGCGTA**AGC**GCCGATGGAAACACAATCGC-3’

In the third step, both the PCR products were mixed in 1:1 molar ratio and end filling was done by PCR in 10 cycles at low annealing temperature. After addition of common forward and reverse primer, PCR was resumed for the next 30 cycles. The final PCR product was digested with XbaI-HindIII and cloned into pTrc99a digested with the same enzymes. The recombinant plasmids, pRB2 (*ldtF*-H135A) and pRB3 (*ldtF*-C143A) were confirmed for the presence of mutations by sequencing.

pRB4. A fragment encoding $LdtF^{20-246}$ was cloned into pET21b vector in between NdeI and XhoI sites using forward and reverse primers

5’-GGAATTCCATATGGGTTTGCTGGGCAGCAGTAG-3 and

5’-CCGCTCGAGTTTTGCCTCGGGGAGCGTGTAG-3’ respectively to generate a C-terminal 6XHis fusion vector. The plasmid was confirmed by sequencing and used for expression and purification of LdtF.

Construction of an *ldtF* deletion mutation (Δ149*ldtF*::Kan)

We constructed a partial deletion mutant of *ldtF* lacking N-terminal 1-149 amino acids using recombineering as described earlier (1). The hybrid primers used for constructing this deletion are:

FP: 5'-

GTCCTGGCGTGTGTAACCGTTTTATCAAGGAATAAGCAGTATGTGTAGGCTGGAG

CTGCTTC-3'

RP: 5'-

CACCAGCGCACCAGTAACGAACTGGAATATCTCATCAATACCATATGAATATCCT

CCTTAG-3'

In the first step, the Kan^R cassette of pKD4 vector (1) was amplified with above set of primers and the purified PCR product was electroporated into a strain encoding λ Red-Gam system. Transformants were selected on plates supplemented with 25 μg/ml kanamycin, followed by confirming the deletion by sequencing and linkage analysis. The deletion was subsequently transferred by P1 transduction into MG1655. This deletion mutant behaved exactly like that of the deletion mutant of Keio collection (in terms of causing sickness to *mepS mepK* double mutant and in PG composition) and hence, Keio deletion mutation was used throughout the study.

Construction of *ldtF*-FLAG fusion

Epitope tagging of LdtF using a 3xFlag was done at the 3′ end of the gene at its native chromosomal locus by recombineering as described earlier (2). The sequence of the hybrid primers used for construction of this fusion is given below:

FP: 5'-
GCAGCCACAACTGGCATCAAACTACACGCTCCCCGAGGCAAAAGACTACAAAGA
CCATGACGGTG-3' and,

RP: 5'-
CGGGCAATGAAACCTGGCAAAAGATTATGCCAGGCGAATGGCGCCATATGAATA
TCCTCCTTAG-3'

The 3xFlag tag along with Kan^R cassette was amplified from plasmid pSUB11 (2) using the above primers. The 5' end of (43 bases) forward primer has homology to the C-terminal of *ldtF* without stop a codon whereas the 3' end (22 bases) has homology to the region encoding Flag epitope of pSUB11. Similarly, the 5' end of reverse primer has a region homologous (42 bases) to the downstream sequence of *ldtF* whereas the 3'end has a region (22 bases) which has homology to the sequence of pSUB11 plasmid at the 3' end. The PCR product was electroporated into DY378 and transformants were selected on plates supplemented with 25 µg/ml Kanamycin at 30°C on LB. The putative *ldtF*-3xFlag-Kan^R region was transferred into MG1655 by P1 transduction and the construct was confirmed by sequencing and linkage analysis. The expression of the fusion tag was confirmed by western blotting using anti-FLAG antibodies.

Confirmation of deletion mutation (mutant from Keio collection) of *ldtF*

The Keio deletion mutant of *ldtF* (3) was confirmed by sequencing the gene-Kan^R junctions. The region encompassing the deletion mutation was amplified using the below primers and sequenced using the same primers.

FP: 5'- GACAGGCTTGCGTAAAACTC-3 and,

RP: 5'- CAGGATGTGGAAATCGACTTCAGC-3

Detailed methods:

Viability assays and Microscopy. Viability of the indicated strains was examined by growing cultures overnight, serially diluting (10^{-2}, 10^{-4}, 10^{-5} and 10^{-6}), and placing 3-5 µl aliquots of each dilution onto the required plates. Plates were normally incubated for 18-24 h at the indicated temperature. To measure growth rates, overnight grown cultures were diluted 1:100 into fresh medium, allowed to grow and OD at 600 nm was determined after every 1 h interval and data were plotted using Origin software. For microscopy, immobilized cultures on a thin 1% agarose pad were visualized using Zeiss AxioImager.Z2 microscope by DIC.

Purification of LdtF

LdtF encoding plasmid, pET21b-*ldtF*$^{20-246}$ (pRB4) was introduced into BL21 (λDE3) strain and transformants were selected on LB plates supplemented with ampicillin (Amp). One purified colony was grown overnight and used to dilute 1:100 into 50 ml fresh LB broth containing Amp. Culture was induced with 250 µM IPTG at 0.6 OD and further allowed to grow for 2 h at 37°C. Cells were harvested, and pellet was stored at −30°C. When required, pellet was resuspended in 1 ml of buffer (50 mM Tris-Cl, 300 mM NaCl and 20 mM imidazole, pH 8.0) and lysed by sonication. Cell debris was removed by centrifugation and the supernatant was mixed with 200 µl Ni^{+2}-NTA agarose (Qiagen) and mixed for 1 h at 4°C. This mixture was loaded onto pre-washed empty column (Bio-Rad) and washed with 30 ml wash buffer-1 (50 mM Tris, 300 NaCl, 30 mM imidazole, pH 8.0) and 20 ml of wash buffer-2 (50 mM Tris, 300 NaCl, 50 mM imidazole, pH 8.0). The bound proteins were eluted with 5 ml of elution buffer (50 mM Tris, 300 NaCl, 150 mM imidazole, pH 8.0) and concentrated to 2.5 ml using a 3 kDa cut-off centrifugal membrane filter (Millipore). This was then loaded onto a buffer exchange PD-10 column and protein was eluted into 3.5 ml 2 x storage buffer (100 mM Tris, 200 mM NaCl, 2 mM DTT). The fraction was further concentrated to 250 µl

using 3 kDa cut-off centrifugal membrane filter and mixed with equal volume of 100% glycerol and stored at −30°C.

Preparation of PG sacculi. Isolation of PG was done as described earlier (4,5). Cells grown to A_{600} of 1.0 were collected by centrifugation at 10,000×g for 10 min at 4°C. Cell pellet (from 1000 ml) was resuspended in 6 ml of ice-cold deionized water and added drop wise into 6 ml of boiling 8% SDS with vigorous stirring followed by boiling for another 45 min to solubilize membranes and to destroy high molecular weight DNA. After overnight incubation at room temperature, the PG sacculi were collected by high speed centrifugation (200,000×g, 40 min) and washed thoroughly with deionized water to completely remove SDS. High molecular weight glycogen and covalently bound lipoprotein, Lpp were removed by treating with α-amylase (100 µg/ml in 10 mM Tris-HCl, pH 7.0, 2 h at 37°C) and pre-digested pronase (200 µg/ml, 90 min at 60°C). Enzymes were inactivated by boiling with equal volume of 8% SDS for 15 min and pure sacculi were obtained by ultracentrifugation and washed several times with water till SDS was completely removed. Final pellet was resuspended in 0.5 ml of 25 mM Tris-HCl (pH 8.0) and stored at −30°C.

Lpp-bound PG sacculi were prepared from cultures grown up to A_{600} of ~6.0 using the above-described protocol except that the treatment with Pronase was not done.

Analysis of PG sacculi. PG analysis was done as previously described (4,5). Essentially, the sacculi were digested with 10 U mutanolysin (Sigma-Aldrich) at 37°C in 25 mM Tris-HCl (pH 8.0) for 16 h and soluble muropeptides were collected after centrifugation. This fraction was reduced with 1 mg of sodium borohydride in 50 mM sodium borate buffer (pH 9.0) for 30 min and excess borohydride was destroyed by addition of 20% phosphoric acid. pH was adjusted to 3–4 and samples were loaded onto a reverse phase C18 column (Zorbax 300 SB; 250×4.6 mm, 5 mm) connected to Agilent technologies RRLC 1200 system. Column

156 temperature was 55°C and binding was done at a flow rate of 0.5 ml/min with 1% acetonitrile
157 in water containing 0.1% trifluoroacetic acid (TFA) for 10 min. Muropeptides were eluted in
158 a gradient of 1–10% acetonitrile containing 0.1% TFA at a flow rate of 0.5 ml/min for next
159 60 min (using RRLC online software called Chemstation). Absorbance of muropeptides was
160 detected at 205 nm.

161 **Mass spectrometry (MS) analysis of muropeptides.** Muropeptide fractions collected during
162 HPLC were dried and reconstituted into 5% acetonitrile with 0.1% formic acid and loaded
163 onto a reverse phase PepMap™ RSLC - C18 column (3 µm, 100Å, 75µmx, 15cm) connected
164 to Q-Exactive™ HF Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher
165 Scientific, USA). Peaks were analysed by tandem MS and structures were decoded based on
166 molecular mass of the individual fragments.

167 **Determination of Lpp-bound muropeptides**

168 Normalized muropeptides were boiled with Laemmli loading dye and separated using 15%
169 SDS-PAGE. Lpp was detected by western blot using rabbit anti-Lpp antibody. As a positive
170 control (free form of Lpp) cell lysates of WT, Δ*ldtF*, or Δ*ldtABC* (equivalent of 0.012 OD)
171 were used.

172 **Cleavage of Lpp from intact PG sacculi**

173 Lpp-bound PG sacculi were isolated from WT and Δ*ldtF* (grown upto ~6 OD) without
174 pronase treatment as described. Equal volumes of the PG sacculi were treated with buffer,
175 pronase (as positive control) or LdtF (4 µM) for 16 h at 30°C. Reaction was stopped by heat
176 inactivation and supernatant was collected after centrifugation at 15000xg for 15 min. These
177 fractions were run on 15% SDS-PAGE to detect the Lpp released from intact PG sacculi. The

178 remaining pellet fraction was further digested with mutanolysin and the resulting soluble
179 muropeptides were separated by RP-HPLC.
180

Supplementary Figures:

Fig. S1

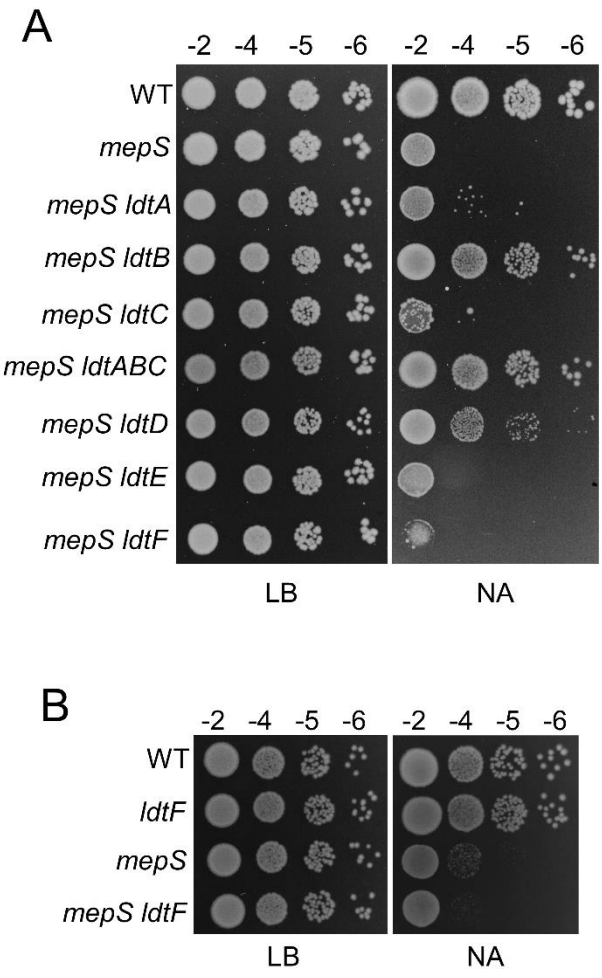
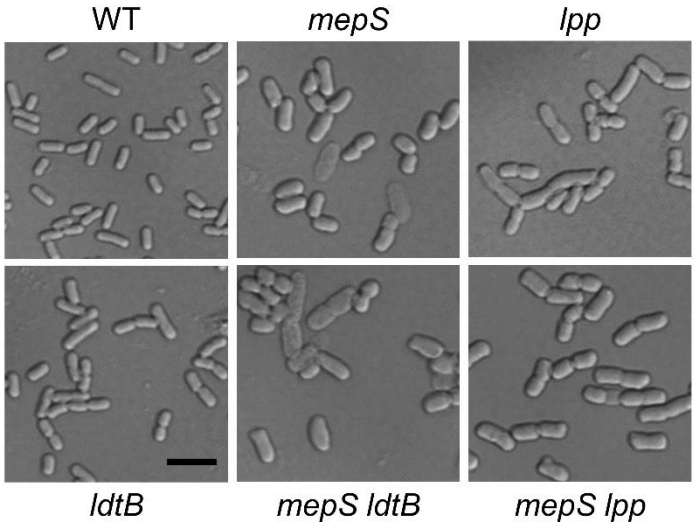


Fig. S1. Genetic interactions of *mepS* with *ldts*. A. WT (MG1655) and its deletion mutant derivatives were grown overnight in LB broth, serially diluted, and 5 µL of each dilution were spotted on indicated plates and tested for viability on NA at 37°C. B. Indicated strains were subjected to viability assays as described above and grown on NA at 30°C.

Fig. S2



188
189 **Fig. S2.** Microscopic images of WT and its various mutant derivatives. Indicated strains
190 were grown overnight and diluted 1:500 into prewarmed Nutrient Broth and grown till A_{600} of
191 1.0 at 37°C and visualized with DIC microscopy. The scale bar represents 5 μm.

Fig. S3

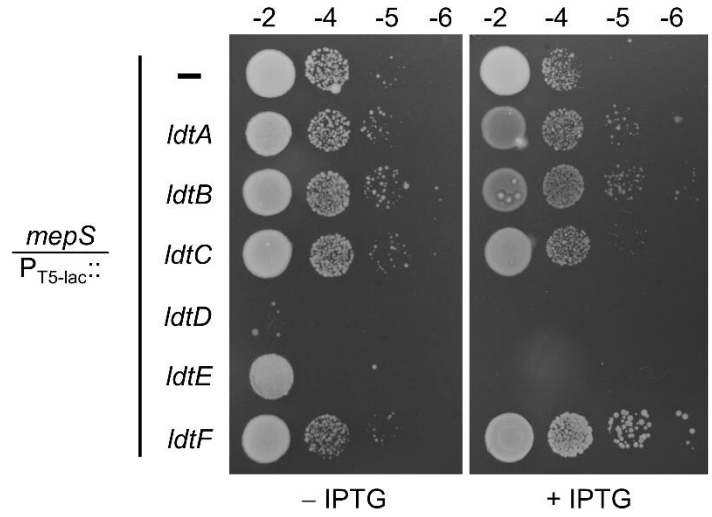
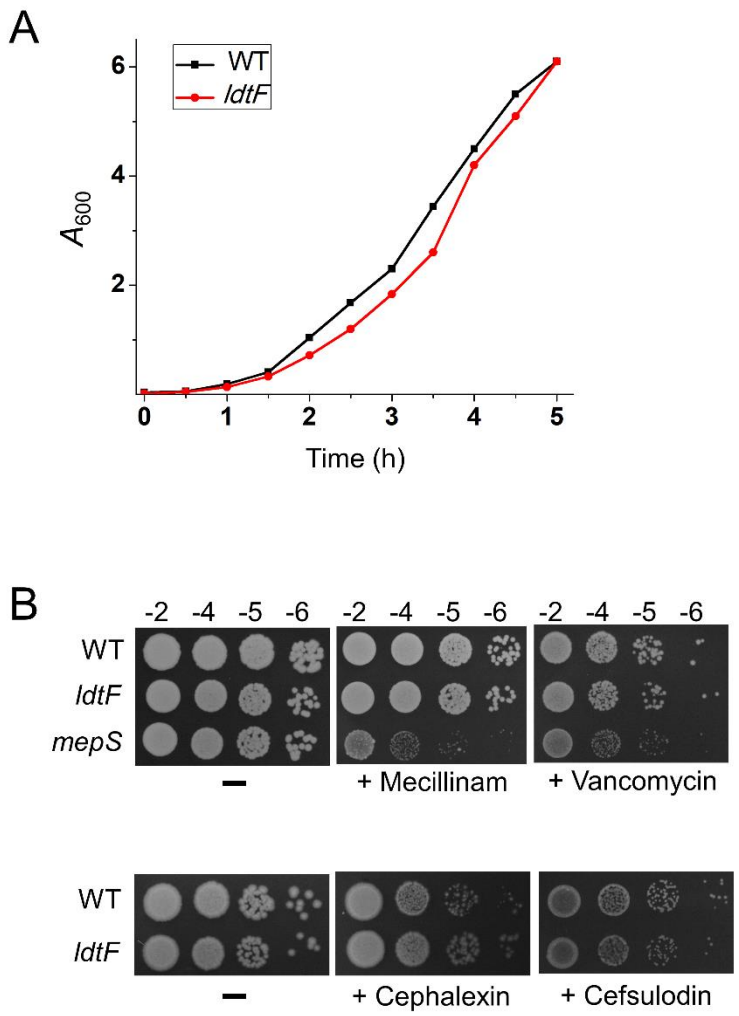


Fig. S3. Effect of overexpression of Ldts on growth of *mepS* mutant. Δ*mepS* mutant carrying either pCA24N vector ($P_{T5\text{-}lac}$::) or its derivatives ($P_{T5\text{-}lac}$::*ldtA-F*) were grown and viability was tested on NA plates at 37°C with or without 50 μM IPTG.

Fig. S4



196

197 **Fig. S4.** Phenotypes of Δ*ldtF* mutant. A. Growth curve of WT and *ldtF* mutant. Overnight
198 grown cultures of WT and *ldtF* were sub-cultured 1:500 into fresh LB and growth was
199 monitored every 30 min at 37°C. B. WT and its mutant derivatives were tested for sensitivity
200 towards mecillinam (0.2 µg/ml), vancomycin (225 µg/ml), cephalexin (4 µg/ml) and
201 cefsulodin (20 µg/ml) on LB at 37°C.

Fig. S5

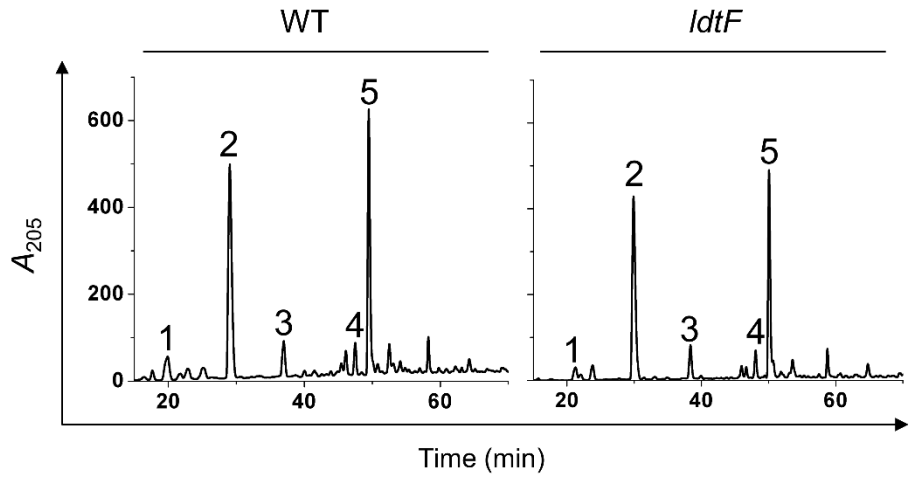


Fig. S5. PG composition of *ldtF* mutant. HPLC chromatograms of PG sacculi isolated from WT and *ldtF* mutant. Strains were grown to an A_{600} of ~1 in LB followed by isolation and analysis of PG sacculi. Data shown is representative of three independent experiments. Peak 1- tri, peak 2- tetra, peak 3- tri-lys-arg, peak 4- tri-tetra, peak 5- tetra-tetra.

Fig. S6

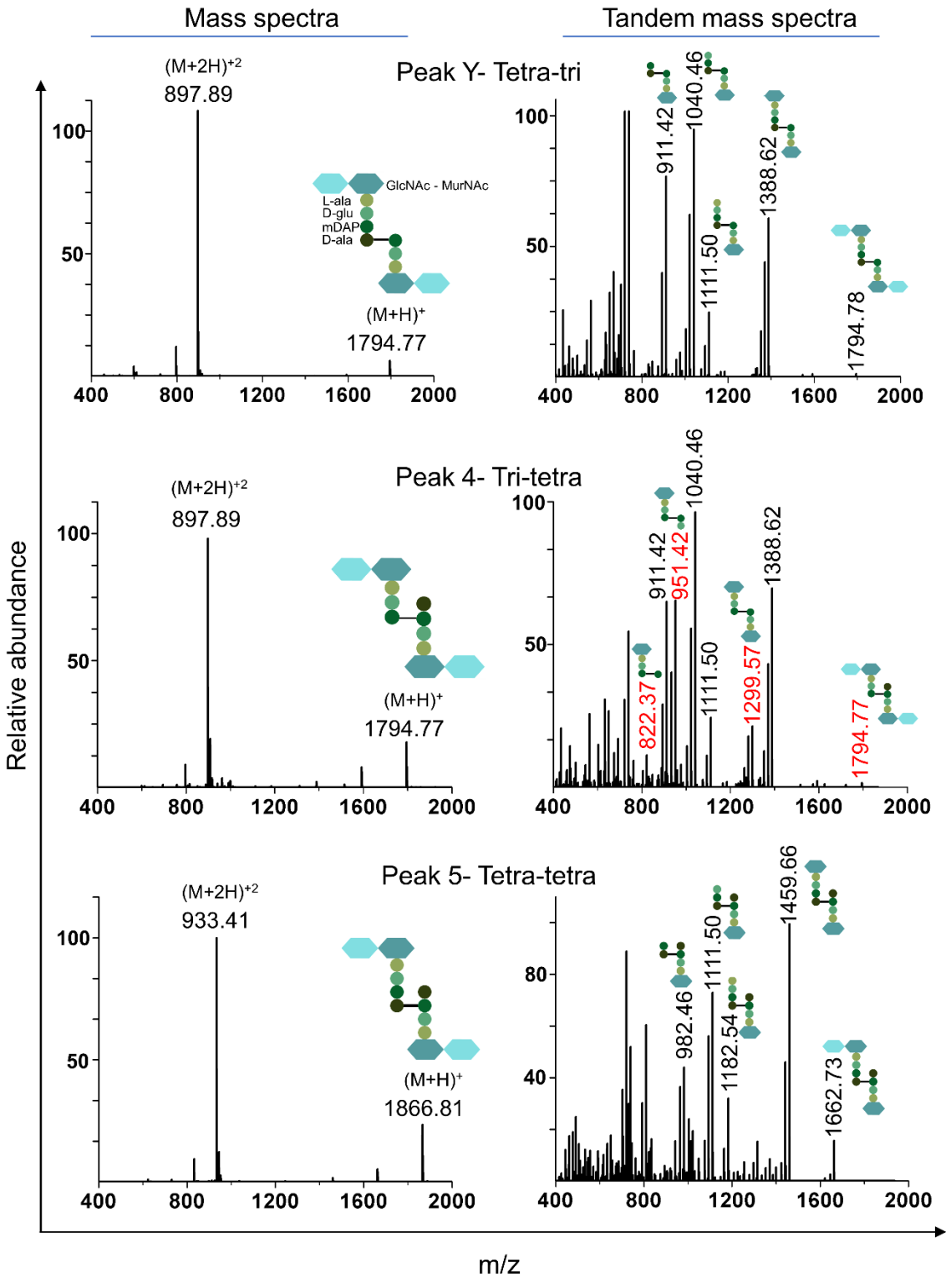


Fig. S6. Mass spectrometry analysis of various muropeptides including tetra-tri, tri-tetra and tetra-tetra. Mass spectra of various muropeptides indicating the molecular mass (both +1 and

+2 charges) is shown. The signature peaks indicating the 3–3 cross-links in tri-tetra are highlighted in red.

Fig. S7

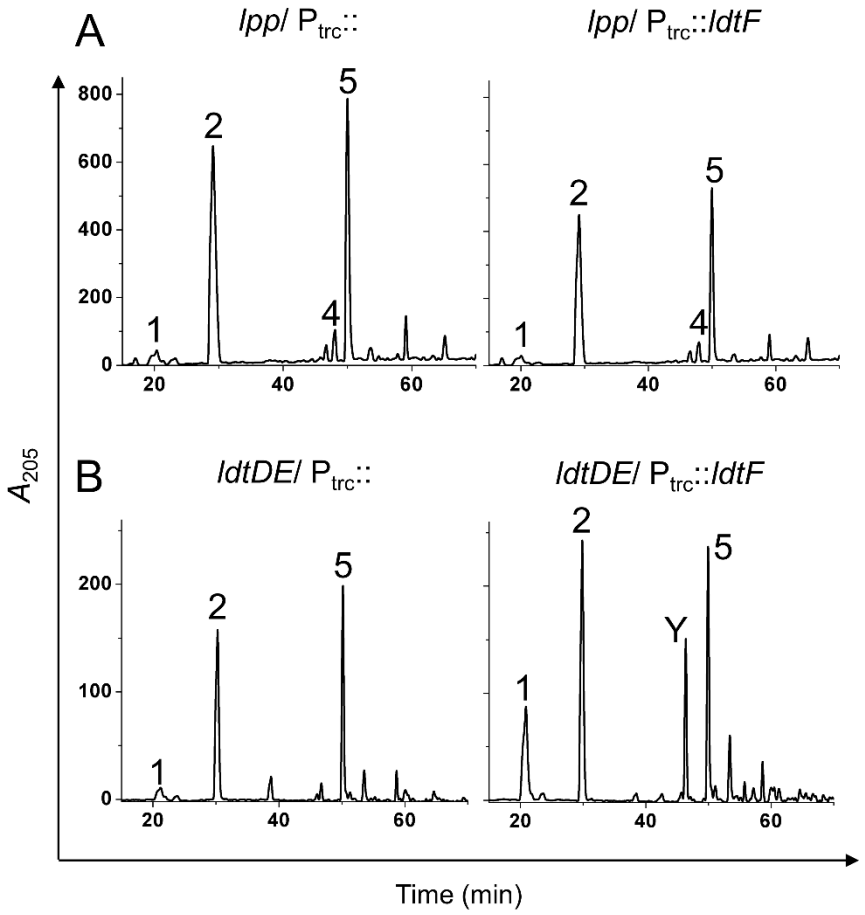


Fig. S7. PG composition of strains with ectopic expression of LdtF. HPLC chromatograms of PG sacculi isolated from (A) *lpp* or (B) *ldtDE* deletion mutants carrying either vector (pTrc99a; P_{trc}::) or pRB1(P_{trc}::*ldtF*). Strains were grown to an A_{600} of ~1 in LB containing 150 µM IPTG followed by isolation and analysis of PG sacculi. Note that in absence of Lpp, increased LdtF does not alter the PG composition. On the other hand, the effect of LdtF is independent of LdtD and -E. Peak 1- tri, peak 2- tetra, peak 3- tri-lys-arg, peak Y- tetra-tri (4-3 crosslink), peak 4- tri-tetra (3-3 crosslink), peak 5- tetra-tetra.

Fig. S8

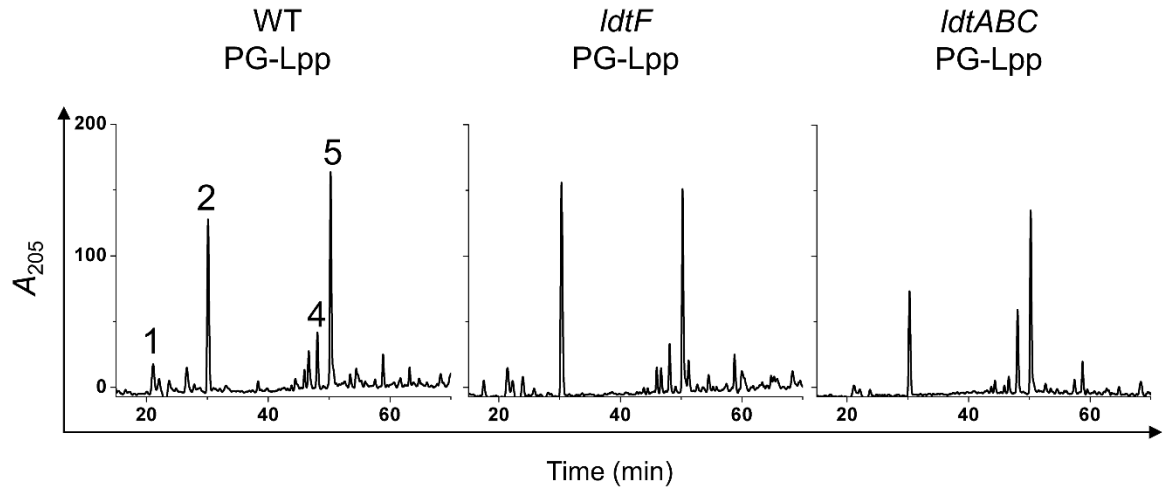


Fig. S8. HPLC chromatograms of Lpp-bound PG sacculi from WT, *ldtF* and *ldtABC* mutant. The data obtained from these chromatograms was used to normalize the amount of sample loaded for performing the experiment described in Fig. 3D. Peak 1- tri, peak 2- tetra, peak 3- tri-lys-arg, peak 4- tri-tetra, peak 5- tetra-tetra.

Fig. S9

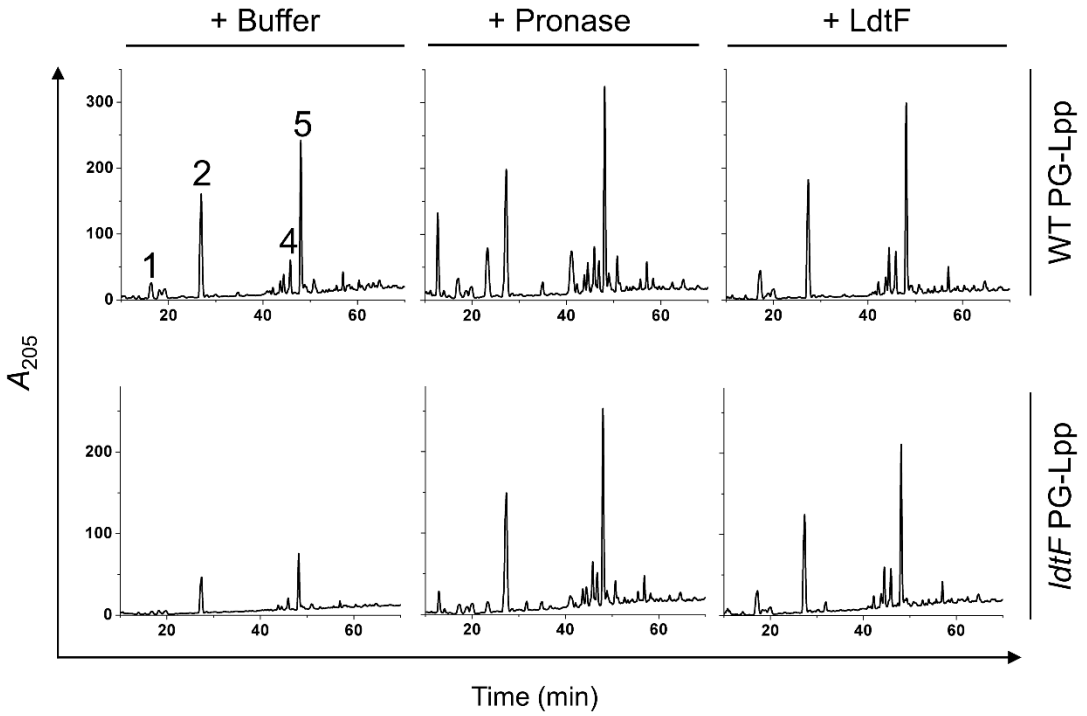


Fig. S9. HPLC chromatograms of Lpp-bound PG sacculi from WT and *ldtF* mutant. The PG sacculi were treated either with buffer, pronase (0.2 mg/ml) or LdtF (4 μM) for 16 h at 30°C and the remaining insoluble fraction was treated with mutanolysin and separated by HPLC. Note that the tri-lys-arg peak is absent in LdtF-treated PG sacculi. Peak 1- tri, peak 2- tetra, peak 3- tri-lys-arg, peak 4- tri-tetra, peak 5- tetra-tetra.

Fig. S10

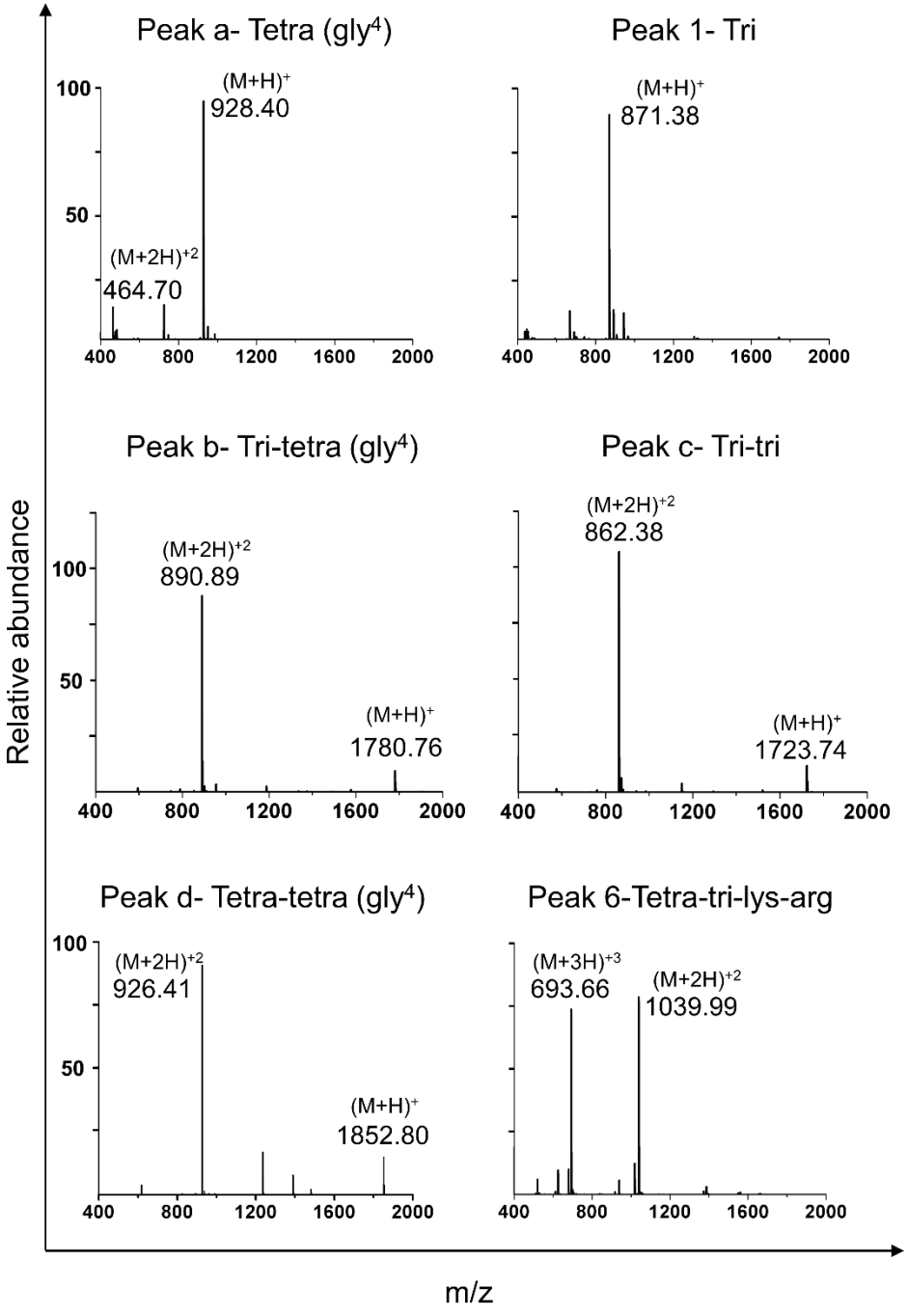
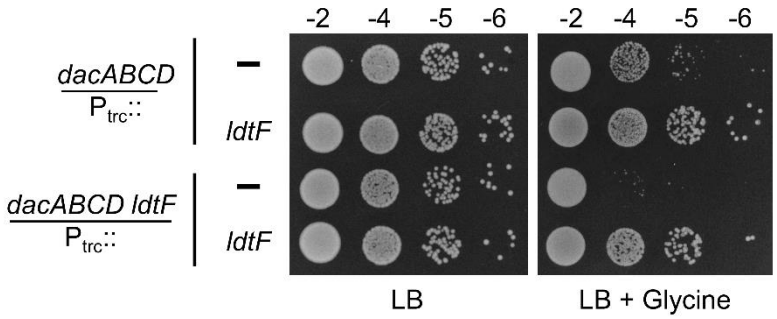


Fig. S10. Identification of muropeptides using Mass spectrometry analysis. Mass spectra of various muropeptides indicating the molecular mass (both +1 and +2 charges) is shown.

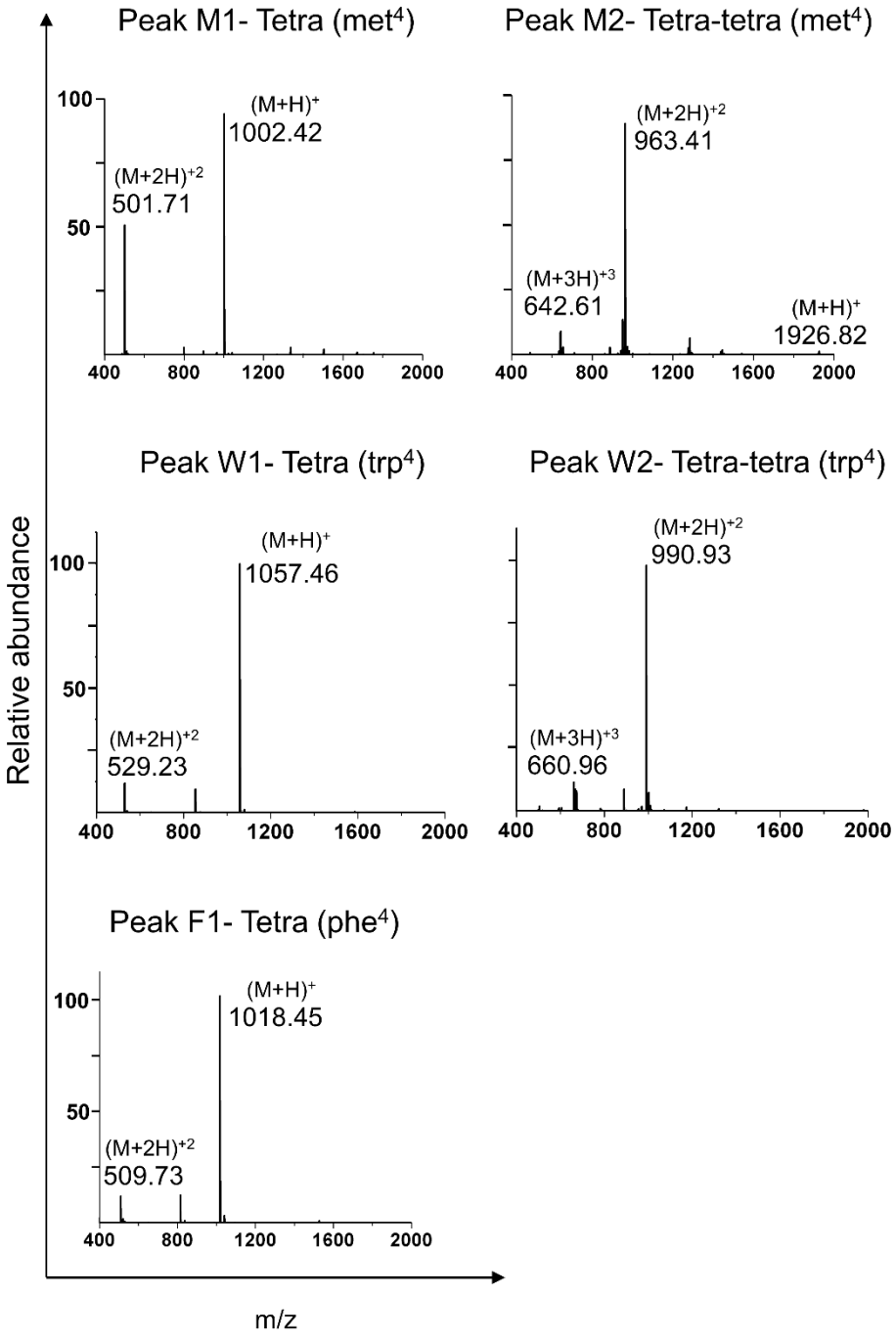
Fig. S11



235

236 **Fig. S11.** Effect of LdtF on glycine-toxicity of *dacABCD* mutant. Cultures of *dacABCD* and
237 *dacABCD ldtF* mutants carrying either vector (pTrc99a; P_{trc}::) or pRB1(P_{trc}::*ldtF*) were
238 grown in LB broth, serially diluted, and 5 µL of each dilution were spotted on indicated
239 plates and tested for viability at 37°C after 18 h of growth. Glycine and IPTG are used at 25
240 mM and 100 µM respectively. *dacABCD* deletion mutant was slow-growing on glycine-
241 supplementation and *ldtF* deletion exacerbated the sickness whereas more copies of *ldtF*
242 improved the growth of the quadruple mutant.

Fig. S12 A



243
244

Fig. S12 B

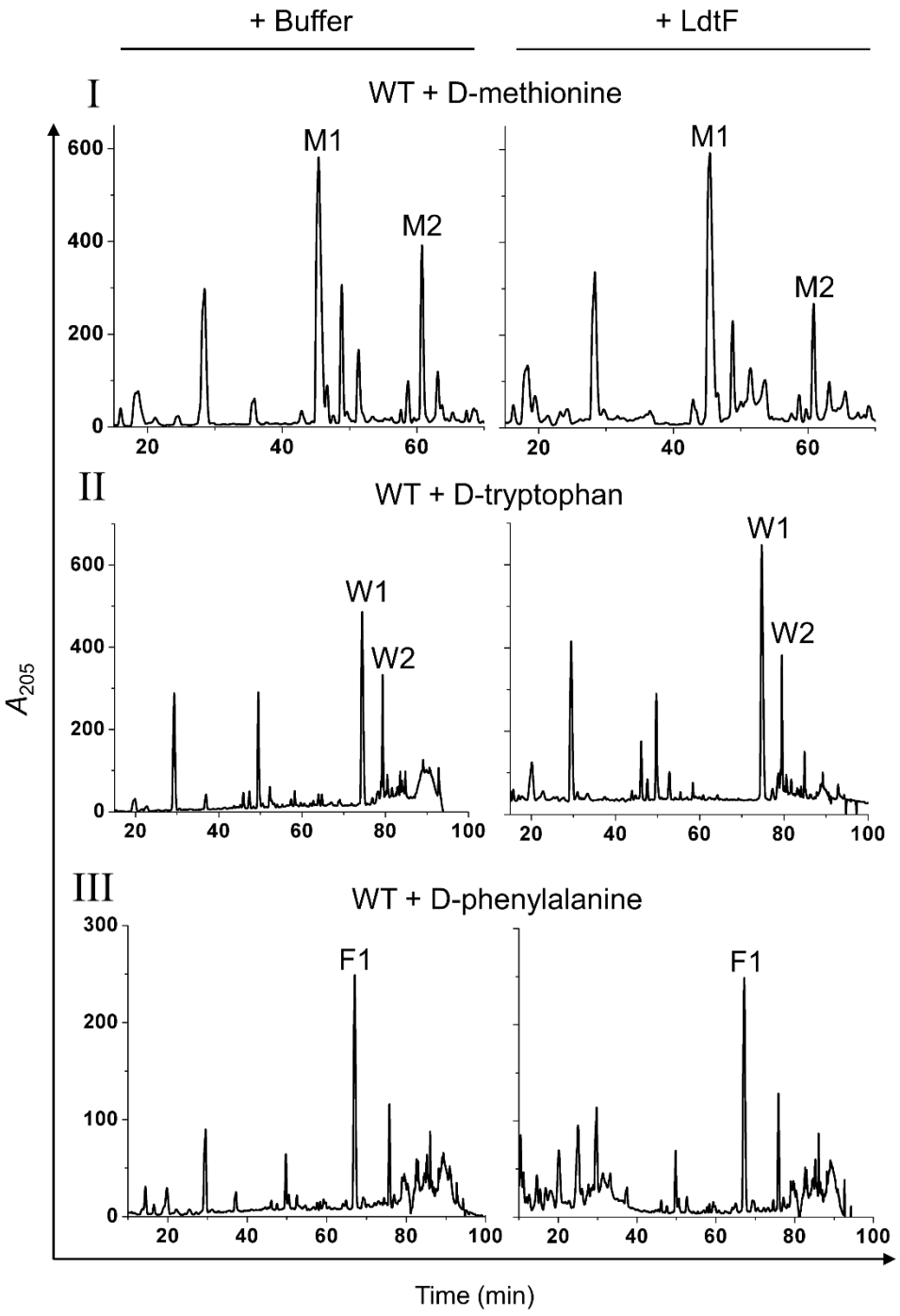


Fig. S12. Effect of LdtF on NCDAA containing muropeptides. (A) Mass spectrometry analysis of muropeptides containing NCDAA (D-methionine, D-tryptophan or D-phenylalanine) (B) Treatment of soluble muropeptides from PG sacculi of strains grown in 20 mM D-methionine (I), 15 mM D-tryptophan (II), or 15 mM D-phenylalanine (III) were

250 incubated either with buffer or LdtF (4 μM) for 16 h and separated by RP-HPLC. LdtF did
251 not cleave the terminal D-amino acids from any of the NCDAA-muropeptides.

252 **Table S1. Strains used in this study**

Strains	Genotype[a]	Source/Reference
WT **(**MG1655)	*rph*1 *ilvG rfb*-50	Lab collection
DH5α	F⁻ *hsdR17 deoR recA1 endA1phoA supE44 thi-1 gyrA96 relA1* Δ*(lac-argF)U169* φ*80dlacZ* ΔM15	Lab collection
BL21 (λDE3)	*ompT rB- mB-* (P_{lac}*UV5*::T7*gene*1)	Lab collection
DY378	Recombineering strain; W3110 *λcI857* Δ(*cro-bioA*)	Lab collection
MR810	Δ*mepS*::*frt*	6
RB11	MG1655 Δ*ldtF*::Kan (Keio)	This study
RB12	Δ*149ldtF*::Kan	This study
RB13	Δ*lpp*::Kan	This study
RB14	[b]$lpp^{\Delta K58}$	This study
RB15	Δ*ldtABC*::*frt*	This study
RB15	Δ*ldtDE*::*frt*	This study
RB16	Δ*dacABCD*::*frt*	This study
RB17	Δ*yciB* Δ*dcrB*	This study

253

254 [a]Deletion alleles used in this study are sourced from Keio collection (3). The deletion
255 mutations were used after testing for their authenticity (by linkage analysis, PCR and
256 sequence analysis) and introduced into different strain backgrounds by P1 phage-mediated
257 transduction (7). MG1655 was used as the wild type strain. The Kan marker from strains was
258 flipped out using pCP20 plasmid encoding a Flp recombinase (1). Flipping creates a '*frt*' scar
259 at the site of deletion.

260 [b]lpp^{K58} allele is from Thomas Silhavy's laboratory. It was transferred by P1 transduction
261 using a 60% linked tetracycline marker.

262

Table S2. Plasmids used in this study

Plasmid	Relevant features	Source/Reference
pET21b	ColE1, Amp^R, *lacI*q, T7*lac*	Lab collection
pTrc99a	ColE1, Amp^R, *lacI*q, P_{trc}	Lab collection
pCA24N	Cm^R, $lacI^q$, $P_{T5\text{-}lac}$	8
pCA24N-*ldtA*	Cm^R, $lacI^q$, $P_{T5\text{-}lac}$::*ldtA*	8
pCA24N-*ldtB*	Cm^R, $lacI^q$, $P_{T5\text{-}lac}$::*ldtB*	8
pCA24N-*ldtC*	Cm^R, $lacI^q$, $P_{T5\text{-}lac}$::*ldtC*	8
pCA24N-*ldtD*	Cm^R, $lacI^q$, $P_{T5\text{-}lac}$::*ldtD*	8
pCA24N-*ldtE*	Cm^R, $lacI^q$, $P_{T5\text{-}lac}$::*ldtE*	8
pCA24N-*ldtF*	Cm^R, $lacI^q$, $P_{T5\text{-}lac}$::*ldtF*	8
pRB1	pTrc99a-*ldtF*	This study
pRB2	pTrc99a-*ldtF*-H135A	This study
pRB3	pTrc99a-*ldtF*-C143A	This study
pRB4	pET21b-$ldtF^{20\text{-}246}$	This study

Table S3. Muropeptide composition of strains carrying multiple copies of *ldtF*

Muropeptide (Peak)	% Area of muropeptide peaks[a] WT/ P_{trc}::	WT/ P_{trc}::*ldtF*	WT/ P_{trc}::*ldtF*$_{H135A}$	WT/ P_{trc}::*ldtF*$_{C143A}$
Tri (1)	5.81 ± 0.06	15.74 ± 0.47	9.51 ± 0.47	8.91 ± 1.41
Tetra (2)	38.91 ± 0.37	34.19 ± 1.70	35.14 ± 1.42	37.6 ± 1.47
Tri-lys-arg (3)	**4.57 ± 0.53**	**0.7 ± 0.60**	**2.2 ± 1.22**	**1.86 ± 1.06**
Tetra-tri (Y)	**3.0 ± 0.23**	**11.75 ± 2.67**	**5.34 ± 0.06**	**5.27 ± 0.77**
Tri-tetra (4)	3.54 ± 0.35	2.47 ± 0.45	3.99 ± 0.25	4.87 ± 0.18
Tetra-tetra (5)	32.22 ± 0.70	25.15 ± 1.79	27.44 ± 2.6	26.33 ± 2.91

[a]Muropeptide analysis was done by calculating the relative percentage area of each muropeptide from the HPLC chromatograms.

References

1. K. A. Datsenko, B. L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 6640–6645 (2000).
2. S. Uzzau, N. Figueroa-Bossi, S. Rubino, L. Bossi, Epitope tagging of chromosomal genes in *Salmonella*. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 15264-15269 (2001).
3. T. Baba, *et al.*, Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**:2006.0008 (2006).
4. B. Glauner, J. V. Höltje, U. Schwarz, The composition of the murein of *Escherichia coli*. *J. Biol. Chem*. **263**, 10088-10095 (1988).
5. P. K. Chodisetti, M. Reddy, Peptidoglycan hydrolase of an unusual cross-link cleavage specificity contributes to bacterial cell wall synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 7825-7830 (2019).
6. S. K. Singh, L. SaiSree, R. N. Amrutha, M. Reddy, Three redundant murein endopeptidases catalyse an essential cleavage step in peptidoglycan synthesis of *Escherichia coli* K12. *Mol. Microbiol.* **86**, 1036-1051 (2012).
7. J. H. Miller, A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY) (1992).
8. M. Kitagawa, *et al.*, Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res.* **12**, 291-299 (2005).