PNAS
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Supplementary Information for

Copper inhibits peptidoglycan LD-transpeptidases suppressing β-lactam resistance due to by-pass of Penicillin-binding proteins

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www.pnas.org/cgi/doi/10.1073/pnas.1809285115

Supplementary Information Text

Supplemental Methods

Peptidoglycan isolation and analysis. *E. coli* strains were grown either in 750 mL TSB media or TSB media supplemented with 0.5 × MIC of $CuCl_2$ (3.75 mM), $CoCl_2$ (2 mM) or $NiCl_2$ (2 mM) to a final OD_{578} of 0.6 at 37°C. For the experiment shown in Fig. S3 (SI Appendix) the BW25113 strain was grown in 500 mL TSB media and in TSB media supplemented with 0.5 × MIC of $CuCl_2$ (3.75 mM) to a final OD_{578} of ~1.0 at 37°C. Cells were cooled on ice for 15 min and collected by centrifugation for 15 min at 4°C and 7,000 × *g*. Cell pellets were resuspended in 7.5 mL cold water and lysed by drop wise addition to 7.5 mL (or 5 mL in case of a starting volume of 500 mL) boiling 8% SDS within 10 min under vigorous stirring. Samples were boiled for further 30 min to ensure complete solubilization of the membranes and degradation of the high molecular weight DNA before storage at 4°C overnight. Samples were heated for 30 min at 60°C and collected by centrifugation 60 min at 90,000 × *g* at 28°C. Pellets were washed once with 7.5 mL (or 5 mL in case of a starting volume of 500 mL) of a 3.75 mM copper-chelating triethylenetetramine dihydrochloride (TETA, Santa Cruz Biotechnology) solution prior to the removal of SDS by several washes with warm water. Further PG preparation was performed as previously described (1). Briefly, muropeptides were released from PG by the muramidase cellosyl (Hoechst, Frankfurt am Main, Germany), reduced by sodium borohydride, and separated on a 250 × 4.6 mm 3 µm Prontosil 120-3-6C18 AQ reversed phase column (Bischoff, Leonberg, Germany). The eluted muropeptides were detected by their absorbance at 205 nm. The PG composition from exponentially growing cells was analyzed in two biological replicates (Fig. 2; SI Appendix, Fig. S4, Table S2 and Table S3), the PG from stationary cells in three biological replicates (SI Appendix, Fig. S3). The muropeptide fraction of TriLysArg was collected and analyzed at the Newcastle University Pinnacle facility as described previously (2).

Optimization of in situ labeling of PG with HADA. We labeled the wt and the Δ6LDT cells with HADA using the published procedure (3, 4). The cells were grown to an exponential phase and a long-term-pulse labeling with the FDAA hydroxyl coumarine-carbonyl amino-D-alanine (HADA) was performed. The excess of dye was removed by 3 washes with 1 × PBS (pH 7.4). The cells were fixed with 3% paraformaldehyde and analyzed under the microscope. Sacculi were extracted from the same cell cultures to ensure that the discovered localization signal of HADA is also visible in the sacculi and to exclude that the detected signals are due to artefacts. Using the published procedure wt cells were completely labeled, but constricting wt cells lacked a signal at the septum, while in Δ6LDT cells no signal could be detected (see Fig. S5A, image 1). Wild-type sacculi showed the same uniform labeling signal and the lack of signal at the septum of constricting cells, while no signal could be detected in Δ6LDT sacculi (Fig. S5B, image 1).

We optimized the procedure with the following washing steps. One-tenth of the final volume of 10 × sodium citrate buffer (pH 2.25) were added to the growing cultures, followed by one wash step with 1 × sodium citrate buffer (pH 3.0) and two wash steps with PBS (pH 7.5). All wash steps were performed at 4°C. Using this optimized protocol wt cells were completely labeled, with a stronger signal at the septum of constricting cells (see Fig. S5A, image 2). The same signal was obtained in purified wt sacculi (Fig. S5B, image 2). Interestingly, in Δ6LDT cells the signals were detected at the septum of constricting cells (Fig. S5A, image 2) and the analysis of the sacculi of those labeled cells confirmed this result (Fig. S5B, image 2). This is realistic, since in the wt strain the HADA incorporation is performed by PBPs and LD-TPases, resulting in the labeled side-wall and in the strong signal at the septum of constricting cells. In Δ6LDT cells HADA is incorporated by the PBPs which are active at the cell septum resulting in the defined localization signal at the septum of constricting cells.

When we grew the wt and Δ6LDT cells in the presence of 0.5 × MIC of $CuCl_2$ (3.75 mM) and performed a long pulse labeling with HADA and removed the excess of dye using 3

washing steps with PBS (pH 7.5) there was a high background signal that impeded the detection of the cellular signal. Adding 10 × sodium citrate buffer (pH 2.25) to the cells prior washing once with 1 × sodium citrate buffer (pH 3.0) and twice with PBS (pH 7.5) improved the detection of the cellular signal, but the background fluorescence was still high.

Therefore we further optimized the washing steps, including one wash step with 7.5 mM triethylenetetramine dihdrochloride (TETA; copper chelating agent) with either pH of 7.5 (Fig. S5A, image 3) or 3.0 (Fig. S5A, image 4). When wt cells were washed once with 7.5 mM TETA pH 7.5 and twice with PBS (pH 7.5) they showed empty septa as in the published procedure (3, 4) while the Δ6LDT cells were unlabeled (Fig. S5A, image 3). By contrary, when the cells were washed once with 7.5 mM TETA pH 3.0 and twice with PBS (pH 7.5) the wt cells showed peripheral and septal labels and Δ6LDT cells a clear septal label (Fig. S5A, image 4).

These results suggest that the washes with low pH buffer (either sodium citrate buffer or TETA solution) improves the removal of excessive HADA, decreasing the background fluorescence signal and thus improves signal detection. In addition, the acidic pH improves the septal signal presumably by preventing the removal of incorporated HADA by DD-carboxypeptidases (5, 6).

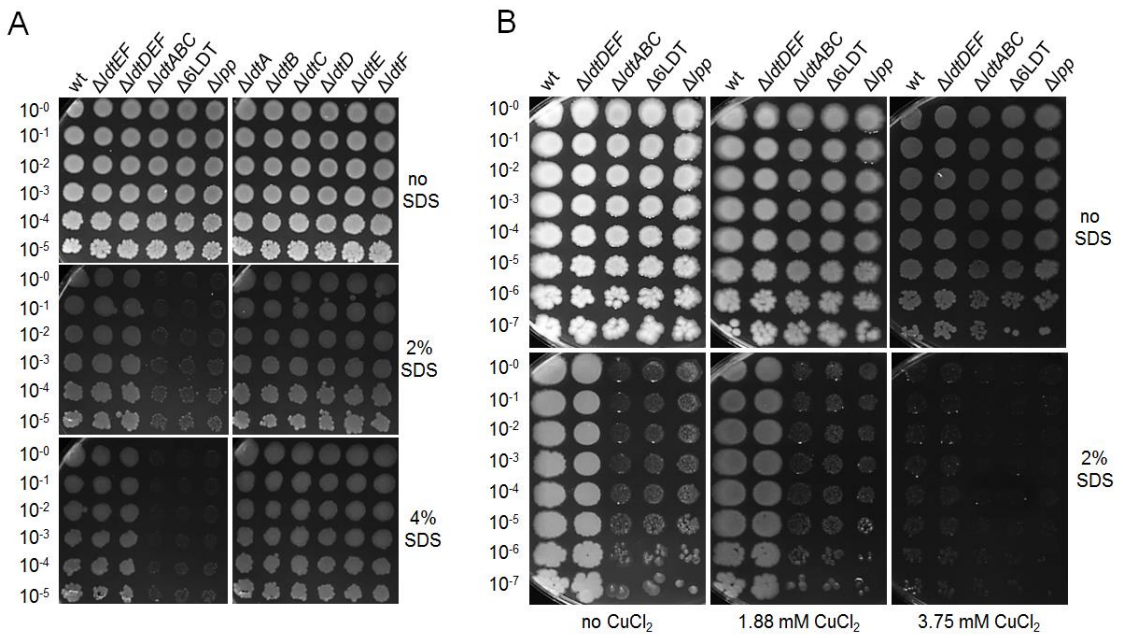

Fig. S1. Sensitivity of *ldt* and *lpp* mutants to SDS and $CuCl_2$. (**A**) SDS sensitivity of *ldt* and *lpp* mutants. Overnight cultures of *E. coli* BW25113 (wt), BW25113Δ*ldtEF*, BW25113Δ*ldtDEF*, BW25113Δ*ldtABC*, BW25113Δ6LDT, BW25113Δ*lpp* and the single LDT mutants BW25113Δ*ldtA*, BW25113Δ*ldtB*, BW25113Δ*ldtC*, BW25113Δ*ldtD*, BW25113Δ*ldtE* and BW25113Δ*ldtF* were adjusted to an equal optical density and serial dilutions were spotted on TSB plates without or with 2 or 4% SDS. Plates were incubated at 37°C for 24 h. Representative results of 3 independent experiments are shown. (**B**) Copper impairs the integrity of the cell envelope. Overnight cultures of *E. coli* BW25113 (wt), BW25113Δ*ldtABC*, BW25113Δ*ldtDEF*, BW25113Δ6LDT and BW25113Δ*lpp* were adjusted to an equal optical density and serial dilutions were spotted on TSB plates with or without 2% SDS, containing no $CuCl_2$, 1.88 mM or 3.75 mM $CuCl_2$ (0, 0.25 and 0.5 × MIC, respectively). Plates were incubated at 37°C for 48 h. Representative results of 3 independent experiments are shown.

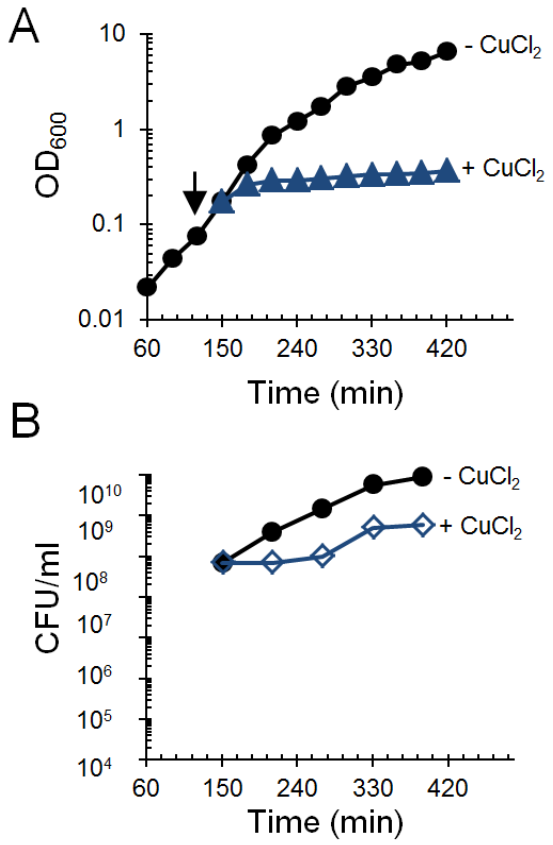

Fig. S2. Growth and viability of the wt strain in the presence and absence of copper. BW25113 cells were grown to an OD_{600} of 0.2 in LD medium. Cells were harvested, washed three times in LD and diluted 1/100 in LD medium. When cells reach an OD_{600} of 0.1 the cultures were split and 3.75 mM $CuCl_2$ was added (arrow). (**A**) Cell growth was monitored by OD_{600} measurements. (**B**) Viability was assessed by determining the colony forming units (CFU).

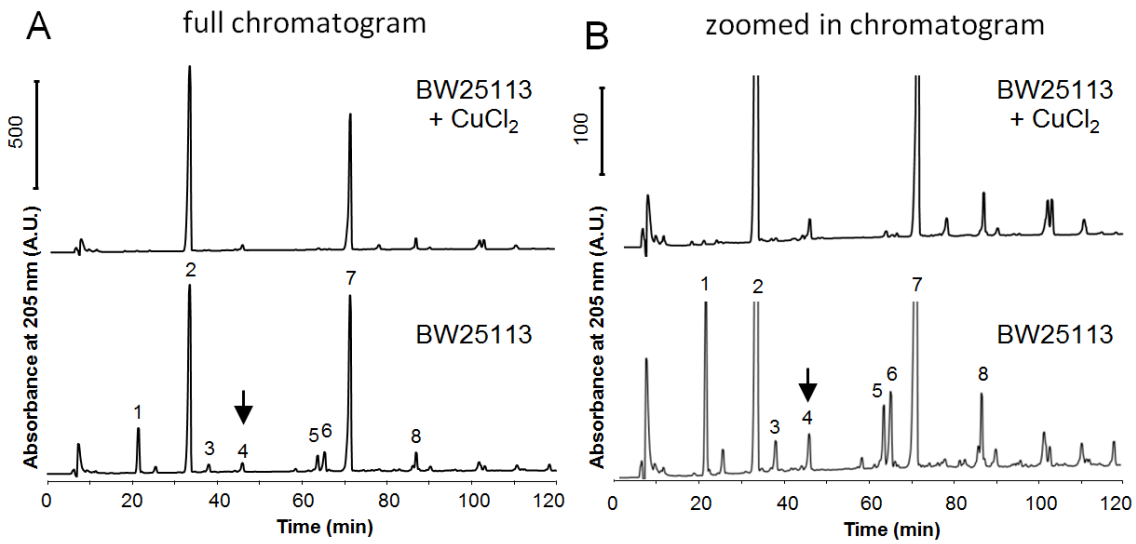

Fig. S3. Effect of copper on the muropeptide composition of stationary cells of *E. coli* BW25113. (**A**) Full muropeptide profiles and (**B**) zoomed in muropeptide profiles of *E. coli* BW25113 grown in the presence of 0.5 × MIC of $CuCl_2$ or without $CuCl_2$. The detected muropeptides are numbered. 1, Tri; 2, Tetra; 3, Di; 4, TriLysArg (arrow); 5, TriTetra(3-3); 6, TetraTri(4-3); 7, TetraTetra(4-3); 8, TetraTetraTetra(4-3). The TriLysArg fraction was collected and the mass of the reduced muropeptide was confirmed by HPLC-LTQ-FT mass spectrometry as previously described (2). Calculated monoisotopic neutral mass: 1154.5667 Da; observed mass: 1154.5676 Da).

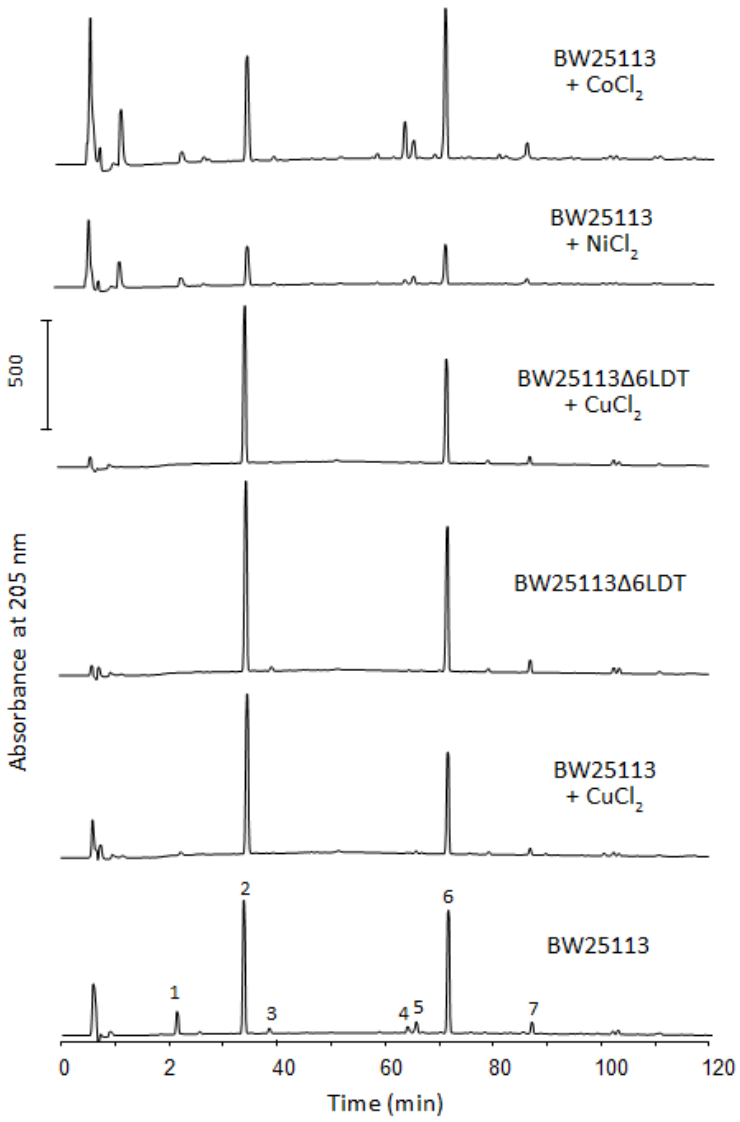

Fig. S4. Effects of metal ions on the muropeptide composition of *E. coli* cells. Muropeptide profiles of BW25113 and BW25113Δ6LDT in the presence of 0.5 × MIC of $CuCl_2$, $NiCl_2$ or $CoCl_2$ or without metal ion. The muropeptides are numbered. 1, Tri; 2, Tetra; 3, Di; 4, TriTetra(3-3); 5, TetraTri(4-3); 6, TetraTetra(4-3); 7, TetraTetraTetra.

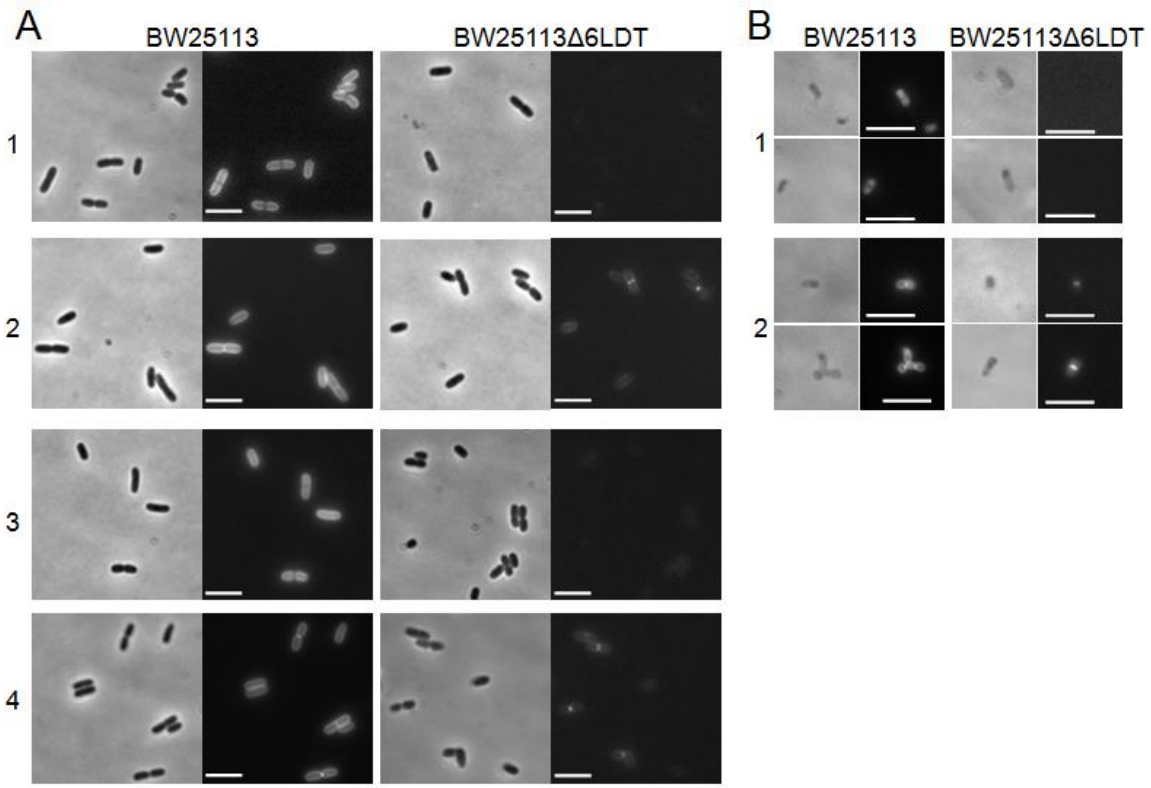

Fig. S5. Optimized HADA labeling of *E. coli* BW25113 and BW25113Δ6LDT cells. BW25113 and BW25113Δ6LDT cells were incubated with 250 µM HADA for 30 min (long pulse labeling). The excess of HADA was removed by different washing procedures (all performed 4°C). 1: 3 washes with phosphate buffered saline (PBS) (published procedure); 2: addition of 1/10 of the final volume of 10 × sodium citrate buffer (805 mM citric acid, 410 mM NaOH, 1.19 M NaCl pH 2.25) to the cell culture prior to one washing step with 1 × sodium citrate buffer (pH 3.0), two washing step with PBS; 3: 1 washing step with 7.5 mM TETA in PBS (pH 7.5), two washes with PBS; 4: 1 washing step with 7.5 mM TETA (pH 3.0) and two washes with PBS. (**A**) After the washing steps cells were fixed with 3% paraformaldehyde. (**B**) Sacculi were isolated from the washed cells and samples applied onto polylysine coated slides. The cells and sacculi were visualized by phase contrast (left) and fluorescence microscopy using the DAPI channel (left). Bar, 2 µm.

Table S1. Bacterial strains.

Strain	Relevant genotype or description[a]	Source or reference
E. coli		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	(7)
AMM28	BW25113Δ*ldtD*::frt Δ*ldtE*::frt Δ*ldtF*::frt, Kan^{S}	A. Polissi
AMM14	BW25113 Δ*ldtA*::frt Δ*ldtB*::frt Δ*ldtC*::frt, Kan^{S}	A. Polissi
BB-3	BW25113 Φ(*kan araC araB*p*lptC*)1	(8)
BW25113Δ6LDT	BW25113 Δ*ldtA*::frt Δ*ldtB*::frt Δ*ldtC*::frt Δ*ldtD*::frt Δ*ldtE*::frt Δ*ldtF*::frt, Kan^{S}	(9)
BW25113Δ*lpp*	BW25113Δ*lpp*::kan, Kan^{R}	(10)
BW25113Δ*erfK*	BW25113Δ*ldtA*::kan, Kan^{R}	(10)
BW25113Δ*ybiS*	BW25113Δ*ldtB*::kan, Kan^{R}	(10)
BW25113Δ*ycfS*	BW25113Δ*ldtC*::kan, Kan^{R}	(10)
BW25113Δ*ycbB*	BW25113Δ*ldtD*::kan, Kan^{R}	(10)
BW25113Δ*ynhG*	BW25113Δ*ldtE*::kan, Kan^{R}	(10)
BW25113Δ*yafK*	BW25113Δ*ldtF*::kan, Kan^{R}	(10)
BW25113 M1	BW25113Δ4 pJEH11(*ldtD*), selection for Amp^{R} in presence of IPTG, Δ*ldtA* Δ*ldtB* Δ*ldtC* Δ*ldtC*, Amp^{R}, CTX^{R}	(11)
BW25113 pTRC99aΩ*bla*$_{TEM116}$	pTRC99aΩ*bla*$_{TEM116}$: *trcP* vector, *luc14,* pUCl8 *EcoR*I-*Hind*III polylinker region, *bla*TEM116 encoding the beta-lactamase (TEM116)	M. Arthur
E. faecium		
D344R	clinical isolate, highly Amp^{R}, Ery^{R}, Tet^{R}, production of low affinity PBP5$_{fm}$	(12)
M512	Amp^{R}, spontaneous mutant derived from D344S by five serial selection steps on agar containing increasing concentrations of ampicillin. D344S is a derivative of D344R lacking the gene encoding PBP5.	(13)

[a]Antibiotic resistance markers: Amp, ampicillin; CTX, ceftriaxone; Ery, erythromycin; Kan, kanamycin; Tet, tetracycline.

Table S2. Effect of copper chloride on the muropeptide composition of *E. coli*.

		Relative peak area (%)[1]			
Muropeptide	**No**	**BW25113**	**BW25113 + $CuCl_2$**	**BW25113 Δ6LDT**	**BW25113 Δ6LDT + $CuCl_2$**
Tri	1	**7.3 ± 0.1**	**0.7 ± 0.7**	**0.0 ± 0.0**	**0.0 ± 0.0**
Tetra	2	42.8 ± 0.0	60.6 ± 0.2	54.4 ± 0.1	60.8 ± 1.4
Di	3	1.8 ± 0.0	0.0 ± 0.0	1.3 ± 0.1	0.0 ± 0.0
TriTetra(3-3)	4	**2.0 ± 0.1**	**0.0 ± 0.0**	**0.0 ± 0.0**	**0.0 ± 0.0**
TetraTri(4-3)	5	**4.1 ± 0.1**	**0.5 ± 0.5**	**0.0 ± 0.0**	**0.0 ± 0.0**
TetraTetra(4-3)	6	38.6 ± 0.3	35.9 ± 1.2	41.1 ± 0.0	36.9 ± 1.2
TetraTetraTetra	7	3.3 ± 0.0	2.4 ± 0.1	3.2 ± 0.2	2.3 ± 0.2

[1]The values are means ± variation of two independent peptidoglycan preparations. Peak areas were quantified with the software Laura (Lab Logic Systems Ltd) and are given as percentage of all known peaks. Bold values highlight muropeptides with tripeptides and/or 3-3 cross-links which are affected due to the presence of $CuCl_2$. Strain BW25113Δ6LDT does not contain tripeptides or 3-3 cross-links.

Table S3. Effect of nickel and cobalt ions on the muropeptide composition of *E. coli*.

		Relative peak area (%)[1]		
Muropeptide	**No**	**BW25113**	**BW25113 + $NiCl_2$**	**BW25113 + $CoCl_2$**
Tri	1	**7.4**	**11.6**	**5.2**
Tetra	2	42.4	38.0	33.5
Di	3	1.8	2.0	1.1
TriTetra(3-3)	4	**2.4**	**4.4**	**9.9**
TetraTri(4-3)	5	**3.9**	**7.6**	**5.8**
TetraTetra(4-3)	6	38.3	32.1	39.5
TetraTetraTetra	7	3.7	4.4	5.0

[1]Peak areas were quantified with the software Laura (Lab Logic Systems Ltd) and are given as percentage of all known peaks. Bold values highlight the changes in muropeptides containing tripeptides and/or 3-3 cross-links in BW25113 cells due to the presence of the respective metal.

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