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From cells to muropeptide structures in 24 h: Peptidoglycan mapping by UPLC-MS

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Peptidoglycan (PGN) is ubiquitous in nearly all bacterial species. The PGN sacculus protects the cells against their own internal turgor making PGN one of the most important targets for antibacterial treatment. Within the last sixty years PGN composition has been intensively studied by various methods. The breakthrough was the application of HPLC technology on the analysis of muropeptides. However, preparation of pure PGN relied on a very time consuming method of about one week. We established a purification protocol for both Gram-positive and Gram-negative bacteria which can be completely performed in plastic reaction tubes yielding pure muropeptides within 24 hours. The muropeptides can be analyzed by UPLC-MS, allowing their immediate determination. This new rapid method provides the feasibility to screen PGN composition even in high throughput, making it a highly useful tool for basic research as well as for the pharmaceutical industry.

The bacterial cell wall is mainly composed of peptidoglycan (PGN) with attached proteins and modifications like wall teichoic acids (WTAs). PGN is a rigid structure of alternating N-Acetylglucosamine-N-Acetylmuramic acid (GlcNAc-MurNAc) glycan chains cross-linked by peptides. The peptide moiety consists of a stem peptide (L-Alanine – D-iGlutamate/iGlutamine – L-Lysin/meso-Diaminopimelic acid – D-Alanine – D-Alanine). L-Lysin (L-Lys) is mainly a feature of Gram-positives, while meso-Diaminopimelic acid (mDAP) is typical for Gram-negatives as well as for *Bacillus subtilis*. We will abbreviate the second amino acid of the stem peptide by Glx which is either glutamine (Gln) or glutamate (Glu). Our model organism *S. aureus* harbors also a five-glycine (Gly_5) interpeptide bridge branching from the L-lysine (Lys), which constitutes indirect cross-links between two adjacent stem peptides[1]. The resulting macromolecule forms a bag shaped sacculus covering the whole bacterial cell. It is unique to bacteria ensuring their shape and protecting them against their own internal turgor. Loss of its integrity results in cell lysis, making PGN one of the most important targets for antibacterial treatment. Indeed, it is the target for well-known therapeutic drugs such as β-lactams or vancomycin, which all inhibit the late steps of glycan chain polymerization and/or cross-linking, both occurring outside the cytoplasmic membrane. With the emergence of new and highly multi-resistant bacterial strains, we are in an urgent need of new anti-microbial drugs. Therefore, it is, amongst others, important to understand the complex interplay between the PGN structure, (changes in) its chemical composition and also subsequent immune responses.

Since its first isolation in 1951 by Salton and Horne[2] PGN composition has been intensively studied with various methods. The original determination of the glycan and peptide composition was performed by paper chromatography[1]. The breakthrough was by Glauner in 1988[3], who for the first time applied high pressure liquid chromatography (HPLC) technology on the analysis of muropeptides. Muropeptides result from enzymatic digestion of the glycan strands into disaccharides, with part of them still being cross-linked by peptides to various extensions. Glauner extensively studied the effects of pH, buffer concentrations, and temperature on HPLC separation of muropeptides of the Gram-negative bacterium *E. coli*. Later, a protocol for PGN isolation and HPLC analysis of the Gram-positive *Staphylococcus aureus* was established by deJonge *et al.*[4]. Basically, these two protocols have been used ever since. However, both of them are very time consuming taking about a week to obtain pure PGN. Both protocols rely on multiple boiling steps with sodiumdodecylsulfate (SDS), which has to be washed out by extensive ultracentrifugation steps for about an hour each. Last year a protocol for isolation of *E. coli* PGN for ultra-performance liquid chromatography (UPLC) analysis was reported, that is substantially

shorter[5]. However, it still relies on washing in an ultracentrifuge thereby limiting the amounts of samples that can be prepared in parallel.

PGN research has experienced a revival within the last decade. Even PGN from bacteria like *Chlamydiae*, which had long been thought to not contain a PGN sacculus, was now successfully isolated and analyzed[6]. In addition, it had been shown, that growth conditions[7,8] as well as various antibiotics as β-lactams and daptomycin affect PGN composition[9–11]. To test PGN composition of different bacteria and under various conditions, a faster analysis method was needed. One which allows to test several conditions in parallel and which can also be applied to bacteria that are not easily grown in high amounts.

We established a purification protocol which can be completely performed in 2 ml reaction tubes, allowing the isolation of up to 48 samples in parallel or even in 96 well plates, yielding pure muropeptides in 24 hours. The muropeptides can be subsequently analyzed by UPLC. This reduces the amount of sample needed and the analysis time from about three hours (HPLC) to 70 min per sample. In addition, the LC conditions were adapted to mass spectrometry (MS) with suitable solvents so that the whole PGN can be directly analyzed by UPLC-MS. This allows for muropeptide determination without first collecting and desalting LC peaks and results in a complete PGN mapping. Taken together, the whole procedure is reduced from about two weeks to 24 hours. Furthermore, this fast isolation method also makes it much easier to obtain larger amounts of PGN which can be used to collect special muropeptides that can then be tested for their ability to stimulate the immune system[12].

Results and Discussion

Peptidoglycan isolation. All steps are performed in 2 ml reaction tubes with a U-shaped bottom. A detailed step-to-step protocol is given in the online method. The method is based on the isolation of PGN by boiling in 1 M NaCl. In the case of some Gram-positive strains we experienced that NaCl treatment is not sufficient. A 0.25% sodium dodecylsulfate (SDS) solution in 0.1 M Tris/HCl (pH 6.8) was then used instead. The SDS has to be washed out again thoroughly. A test for residual SDS is available and should be performed[13]. The resulting cell walls are washed with water and broken into smaller fragments by sonication. DNAse, RNAse, and trypsin are used to digest residual nucleic acids as well as cell wall bound proteins. The enzymes are inactivated by boiling and the cell walls are washed again with water. Treatment with 1 N HCl releases bound wall teichoic acids (WTA) or other glycophosphates. Higher concentrations of HCl must be avoided, as they result in clumping of the sample. In former protocols[4], 48% HF was used to release WTA, but comparison of PGN treated with HCl or with HF showed no significant differences (see Fig. S1). Washing the pellet until the pH is neutral results in pure PGN that can be digested by cell wall hydrolases. In our case the PGN was digested with mutanolysin for 16 hours at 37°C shaking.

Peptidoglycan analysis by UPLC. Prior to UPLC analysis the MurNAc residues of the muropeptides are reduced into NAc-muraminitol by sodium borohydrate ($NaBH_4$). Otherwise, mutarotation of the non-reduced glycan end would result in double peaks. Analysis is performed by UPLC (or HPLC) using trifluoracetic acid (TFA) and a methanol gradient from 5 to 30% (for Gram-positives) or from 0 to 30% (for Gram-negatives) in 70 min. TFA is an ion pairing agent that forms an ion bond with the muropeptides resulting in a good separation of mixed analyts. As TFA is volatile it can also be used for MS analysis. However, it reduces the sensitivity of the mass spectrometer which might require extra washing after use. After testing several different columns, the reversed phase UPLC column CSH C18, 130Å, 1.7 μm, 2.1 mm × 100 mm of Waters was best suited for our needs.

Peptidoglycan mapping by UPLC-MS. The samples can also be directly analyzed by UPLC-MS. We used a Synapt G2 mass spectrometer coupled to a Waters Acquity H-class and operated in positive ESI mode with a scan range from 50–2,000. The data was analyzed by MassLynx. As an example for PGN isolation by this new method, UPLC analysis and MS-mapping are presented in Fig. 1 and Fig. 2. For Gram-positives we used *Staphylococcus aureus* (*S. aureus*) SA113[14] and for Gram-negatives *Escherichia coli* (*E. coli*) Nissle 1917[15].

Exemplary analysis of *S. aureus* SA113 by UPLC and UPLC-MS. The methicillin sensitive *S. aureus* strain SA113 was grown over night in BM medium. We isolated its PGN and analyzed it by UPLC as well as by UPLC-MS. This enabled us to directly determine the masses of all muropeptide peaks we got by UPLC separation. The total ion current (TIC) chromatogram obtained by UPLC-MS was almost identical to the UV pattern of UPLC alone with two exceptions: 1) Retention time of the peaks in the quaternary UPLC was about 1 min longer than in the binary UPLC-MS system. Therefore, in tables 1 and S1 only the TIC retention times are given. 2) The signal of some muropeptides, that turned out to be O-acetylated, were stronger in the TIC than in the UV (e.g. peaks 9 in Fig. 1a and b).

The UV chromatogram of the UPLC as well as the TIC chromatogram of the MS show the expected pattern as it had been published before for the methicillin resistant *S. aureus* COL by de Jonge *et al.* in 1992[4,16]. By coupling UPLC to MS we could directly determine the masses and subsequently the potential structures of almost all peaks. The obtained masses verified that the main peaks contained the expected muropeptides starting with the monomeric peak 5 (Penta(Gln)Gly_5) which is the basic structure of all muropeptides. Peaks 11, 15, 16, 17 and 18 were composed of this basic structure cross-linked by the Gly_5 interpeptide bridge into dimers, trimers, tetramers, pentamers, and hexamers, respectively (for general structures see Fig. 3). This proved that our substantially shortened purification method as well as the adaption of UPLC-MS is working properly. Tab. 1 gives an overview on the muropeptides of the main peaks that we detected in our analysis which had already been determined before[4,17]. The complete results of our PGN mapping of *S. aureus* SA113 is given in Tab. S1. All structures were drawn by ChemDrawUltra 13.0 (PerkinElmer), which automatically calculated the mass of the molecule and the proposed sum formula. The latter is given. As can be judged by the peak forms in the UPLC chromatogram, most peaks are composed of several muropeptides. While all of them have the same basic structure (MurNac-GlcNac with stem peptide and Gly_5 interpeptide bridge), several modifications occur, which will be discussed in detail:

The lengths of the stem peptides are variable. Monomeric stem peptides of *S. aureus* are mostly composed of the following five amino acids (L-Ala – D-iGln – L-Lys– D-Ala – D-Ala) with the D-iGln resulting from the amidation of the original D-iGlu[18,19]. These structures are called "Pentas". Cross-linking of several muropeptides by their interpeptide bridges forms multimeric muropeptides with normally one Penta and several Tetras, as the last D-Ala is cleaved off during the transpeptidation reaction. The resulting muropeptides are called "Penta-$Tetra_n$". However, several of the higher cross-linked muropeptides had lost the last D-Ala on the Penta stem peptide shortening it to a Tetra, forming "$Tetra_n$" muropeptides. No further degradation into tripeptides was observed suggesting that *S. aureus* does not possess an L,D-carboxypeptidase activity.

In *S. aureus*, the D-iGlu at position two of the stem peptide is almost quantitatively amidated resulting in D-iGln[18,19]. However, some muropeptides still harbor the original D-iGlu. While these non-amidated forms have been published to have a shorter retention time than their amidated counterparts[18], in our system they had

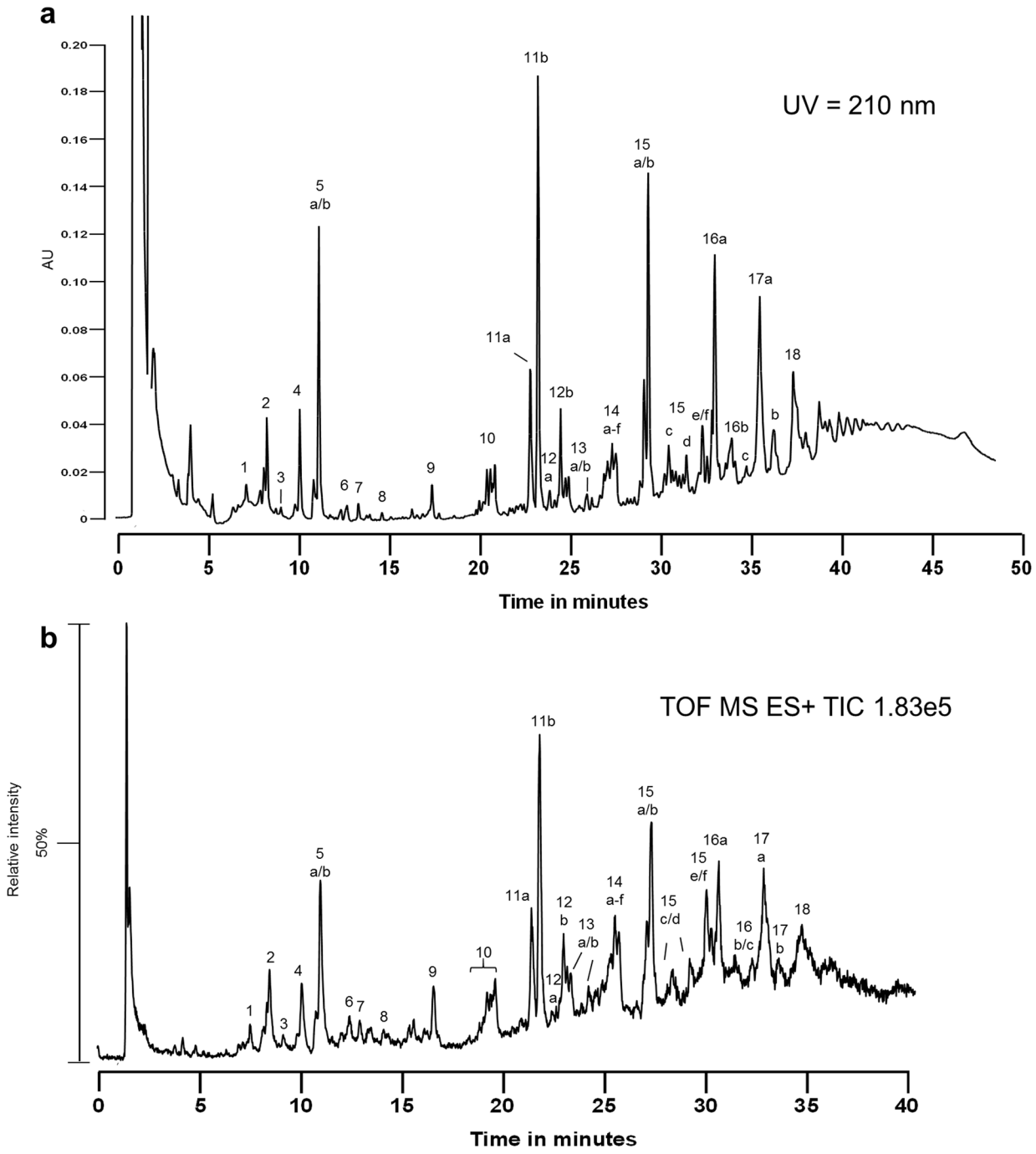

Figure 1 | **Muropeptide profile of *S. aureus* SA113 obtained by UPLC and UPLC/MS.** (a) Muropeptide profile of *S. aureus* SA113 obtained by reversed phase UPLC. (b) TIC of UPLC/MS analysis of *S. aureus* SA113 obtained by reversed-phase UPLC coupled to MS. Masses of indicated peaks are shown in table 1 and table S1 including molecule composition and proposed sum formula.

longer retention times. This might be due to the different column used for analysis.

The length and the composition of the interpeptide bridge varies. Gly numbers that are not a multiple of 5 indicate that this very muropeptide was originally part of a bigger molecule but had been reorganized within the PGN sacculus. For example we found monomeric muropeptides with 6, 7, 8 or 9 Gly residues instead of 5, and dimeric muropeptides with 6, 7, 8 or 9 Gly residues instead of 10. In Tab. 1 and Tab. S1 the sum of all Gly residues of each muropeptide is given. In addition, two muropeptides contained Ala in exchange for one Gly in the interpeptide bridge. Muropeptides harboring various numbers of Gly residues or Ala in their interpeptide bridge have been published before[4].

There also occur changes in the saccharide moiety. Several muropeptides had lost one GlcNAc residue. In rare cases we also found an additional GlcNAc residue, which resulted in a muropeptide with a trisaccharide, but we did not observe the addition of an extra MurNac. In table S1 the addition or loss of a GlcNAc residue is given as +1 or −1 respectively.

Even though O-acetylation had been thought to be lost during acidic treatment, we detected that most of the small peak groups, as well as some of the peak shoulders, both of which had not been analyzed so far, were composed of acetylated muropeptides. Even treatment with HF did not result in a loss of the O-acetylated muropeptides (see Fig. S1). As an example, peak 9 contained only the O-

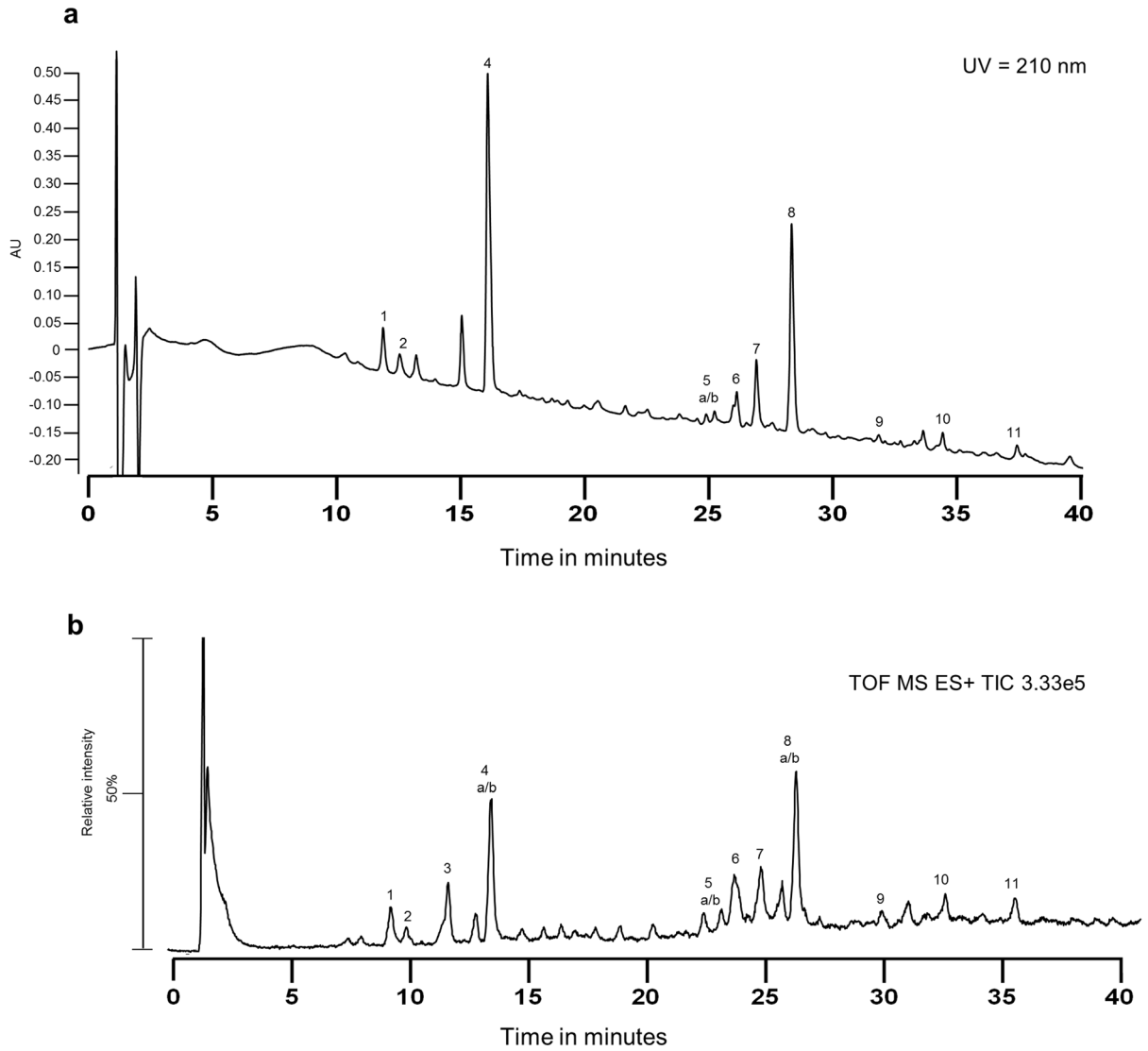

Figure 2 | **Muropeptide profile of *E. coli* Nissle 1917 obtained by UPLC and UPLC/MS.** (a)Muropeptide profile of *E. coli* Nissle 1917 obtained by reversed phase UPLC. (b) TIC of UPLC/MS analysis of *E. coli* Nissle 1917 obtained by reversed-phase UPLC coupled to MS. Masses of indicated peaks are shown in table 2 and table S2 including molecule composition and proposed sum formula.

acetylated monomer Penta-Gly_5. If HF caused de-O-acetylation, this peak should be missing in Fig. S1B, but it is not. The PGN of pathogenic staphylococcal strains is highly O-acetylated on the 6th C-atom of the MurNAc, which renders the bacterium resistant against lysozyme[20]. Lysozyme is an enzyme produced by humans and animals as a defense mechanism. It hydrolyzes PGN and therefore kills bacteria.

Table 1 | Muropeptides of *S. aureus* SA113 analyzed by UPLC-MS

		M+H$^+$			Inter-peptide bridges		
Peak	Reten-tion time in TIC [min]	Measured	Proposed sum formula	Lengths of the stem peptides	Gly	Ala	Prev. pub.
2	8.56	1239.5792	C48H83N14O24+	Tetra	6		4
4	10.17	1025.5032	C41H73N10O20+	Penta	1		4
5b	11.08	1253.5861	C49H85N14O24+	Penta	5		4
8	14.20	1267.6096	C50H87N14O24+	Penta	4	1	4
11b	21.93	2417.1181	C95H162N27O46+	Penta-Tetra	10		4
12b	23.12	2328.0745	C92H155N26O44+	$Tetra_2$ (cyclic)	10		17
15b	27.45	3580.6630	C141H239N40O68+	Penta-$Tetra_2$	15		4
16a	30.80	4744.1962	C187H316N53O90+	Penta-$Tetra_3$	20		4
17a	33.01	5907.7014	C233H393N66O112+	Penta-$Tetra_4$	25		
18	34.90	7071.2313	C279H470N79O134+	Penta-$Tetra_5$	30		

Each muropeptide contains one to six stem-peptides, each consisting of L-Ala – D-Gln – L-Lys – D-Ala (– D-Ala). The amount of Gly molecules building the interpeptide bridges is given as the sum of all Gly residues present in each muropeptide. In rare cases one Gly is replaced by an Ala. The last column refers to the original reference of the respective muropeptide.

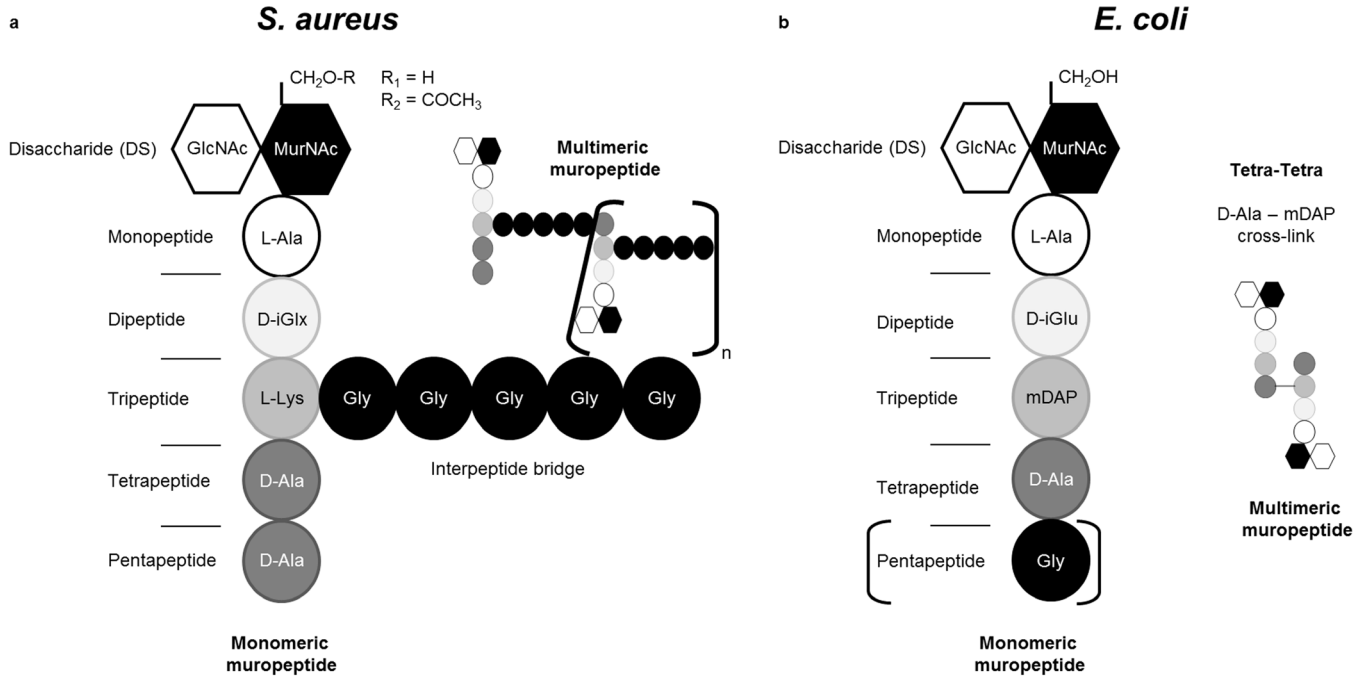

Figure 3 | **Schematic structure of muropeptides of *S. aureus* and *E. coli*.** The basic structure consists of the disaccharide (GlcNAc-MurNAc) with an adjacent stem peptide (L-Ala – D-iGlx – L-Lys – D-Ala – D-Ala) with Glx being either Gln or Glu. Our model organism *S. aureus*, harbors also a five-glycine interpeptide bridge branching from the L-Lys, which constitutes indirect cross-links between two adjacent stem peptides, as it is depicted in the multimeric muropeptide. The C at position 6 of the MurNAc can be O-acetylated.

Even though we cannot exclude that part of the O-acetylation is lost during isolation, a change of the respective peaks would be an indication for changes in the O-acetylation grade and could be useful to screen for possible mutants or for drugs affecting O-acetylation.

A very small amount of MurNac residues seemed not to be completely reduced to NAc-muraminitol. This also resulted in longer retention times compared to the reduced counterparts.

Exemplary analysis of *E. coli* Nissle 1917 by UPLC and UPLC-MS. As an example for Gram-negative bacteria we grew *E. coli* Nissle 1917 overnight in LB medium and isolated its PGN. The PGN was digested by mutanolysin and analyzed by UPLC and UPLC-MS (Fig. 2). Again, the UV pattern was very similar to the TIC chromatogram and we could determine the masses of most muropeptide peaks. The masses we found resulted in proposed muropeptide structures that are in accordance with the ones previously published by Glauner[3] (Tab. 2). The complete analysis is given in Tab. S2.

We found the expected monomeric muropeptides with the Tri and the Tetra stem-peptide (peak 1 and 4, respectively), the dimeric muropeptides Tetra-Tri and Tetra-Tetra (peaks 7 and 8, respectively) as well as in small amounts the trimeric muropeptide Tetra-Tetra-Tetra (peak 10). (For a schematic drawing of the Gram-negative muropeptides see Fig. 3b). Interestingly, for the Tetra, the Tetra-Tri and the Tetra-Tetra, we always got two peaks in the TIC chromatogram but only one in the UV chromatogram. We fragmented the respective peaks and they seem to be stereo-isomers as in all

Table 2 | Muropeptides of *E. coli* Nissle 1917 analyzed by UPLC-MS

Peak	Reten-tion time in TIC [min]	M+H+ Measured	Proposed sum formula	Length of the stem peptides	Prev. pub.
1	9.27	871.3793	C34H59N6O20+	Tri	3
2	9.95	928.3986	C36H62N7O21+	Tetra-Gly(4)	3
3	11.69	699.2936	C27H47N4O17+	Di	3
		999.4775	C39H67N8O22+	Penta-Gly(5)	3
4a	12.81	942.4180	C37H64N7O21+	Tetra	
4b	13.50	942.4177	C37H64N7O21+	Tetra	3
5a	22.40	1851.8278	C73H123N14O41+	Tetra-Tetra-Gly(4)	
5b	23.10	1851.7966	C73H123N14O41+	Tetra-Tetra-Gly(4)	3
6	23.60	1922.8710	C76H128N15O42+	Tetra-Penta-Gly(5)	3
		1794.7766	C71H120N13O40+	Tetra-Tri	3
7	24.70	1794.7758	C71H120N13O40+	Tetra-Tri	3
8a	25.59	1865.8140	C74H125N14O41+	Tetra-Tetra	
8b	26.18	1865.8140	C74H125N14O41+	Tetra-Tetra	3
9	29.75	2846.2697	C113H189N22O62+	Penta-Gly(5)-Tetra-Tetra	
10	32.40	2789.2146	C111H186N21O61+	Tetra-Tetra-Tetra	3
11	35.30	1845.7744	C74H121N14O40+	anhydro Tetra-Tetra	3

Each muropeptide contains one to three stem-peptides, each consisting of L-Ala – D-Glu – mDAP – D-Ala. In some cases the D-Ala on position four is replaced by a Gly. Some muropeptides contain Penta stem-peptides with Gly on position five. The last column refers to the original reference of the respective muropeptide.

three cases closely related fragmentation spectra with different relative fragment peak intensities were obtained. Fig. S2 gives the exemplary fragmentation of peaks 8a and 8b, both being the Tetra-Tetra muropeptide. We did not get any hints for mDap-mDap-crosslinking, as it had been seen in strain *E. coli* W7[3].

The monomeric peak 3 in the TIC chromatogram is not in correlation with any peak of the UV chromatogram. Vice versa, the UV chromatogram has two unlabeled peaks, for which we cannot assign a muropeptide. Peak 3 of the TIC chromatogram contains 4 muropeptides (Tab. S2) with two of them being already known from the Glauner analysis[3], the Di and the Penta-Gly(5) monomer. While these two peaks in the Glauner analysis have a higher retention time than the Tetra, they have a shorter retention time when analyzed with our conditions. The same is seen for Tetra-Penta-Gly(5) (peak 6) which in our system elutes before the Tetra-Tri and not after it.

With these few exceptions the elution profile of the *E. coli* muropeptides under our conditions is the same as already known. We also found one anhydro Tetra-Tetra muropeptide (peak 11), but we did not find any structures still containing lysine and arginine, which would be remnants from Braun's Lipoprotein[21]. Instead we found masses corresponding to muropeptides which had lost the GlcNAc moiety (Tab. S2). These are probably features of the strain we used. As expected, in all muropeptides with a Penta stem-peptide, the amino acid in position five is a Gly, as the last D-Ala is cleaved off by the D,D-carboxypeptidase penicillin-binding protein (PBP) 5[22]. We did obtain masses for the peaks between minutes 14 and 21, but they did not fit any muropeptide structures and are therefore not given.

Conclusion

We have presented here a very quick method for PGN isolation with additional analysis on UPLC-MS all within 24 hours. This method is suitable for high throughput screening of various bacterial strains or growth conditions, as it is performed in either 2 ml plastic reaction tubes or even in microtiter plates. With this new procedure we mapped the whole PGN of *S. aureus* SA113 up to the hexameric cross-linked muropeptide. In addition to the already known muropeptide structures of the prevalent peaks we have also presented masses and structures for the so far unidentified smaller peak groups in between. As an example for Gram-negatives we analyzed the PGN of *E. coli* Nissle 1917 and corroborated former publications.

Methods

The protocols will also be provided at www.nature.com/protocolexchange.

Bacterial strains. *S. aureus* SA113[14] and *E. coli* Nissle 1917[15].

Cell growth. The cells were grown in their respective medium to the needed OD. Basic medium (BM) for *S. aureus* consisted of Soy Peptone (10 g; Plato), Yeast Extract (5 g; Deutsche Hefewerke), NaCl (5 g; Carl-Roth), Glucose (1 g; Carl Roth) and K_2HPO_4 (1 g; Applichem). Deionized water was added to a final volume of 1 liter and pH was adjust to 7.2. LB medium for *E. coli* consisted of Peptone (10 g; Plato), Yeast Extract (5 g; Deutsche Hefewerke) and NaCl (5 g; Carl Roth). Deionized water was added to a final volume of 1 liter and pH was adjust to 7.2.

Reagents. Unless otherwise stated, all reagents were bought from Sigma-Aldrich.

Midipreparation for UPLC/MS or HPLC/MS analysis. This protocol results in sample amounts suitable for several analyses. From 2 ml of culture of $OD_{578} \approx 10$ about 300 µl purified PGN are gained. Spin down 2 ml of an overnight culture in a 2 ml microcentrifuge tube (Eppendorf) for 5 min at 10,000 rpm. Alternatively: Spin down 2 × 2 ml of a culture with a lower OD. Resuspend the pellet in 1 ml solution A (1 M sodium chloride) and boil the suspension for 20 minutes at 100°C in a heating block. [Δ CRITICAL STEP 1: Sometimes, NaCl treatment is not sufficient for peptidoglycan isolation from the cells. Use 0.25% SDS solution in 0.1 M Tris/HCl (pH 6.8) instead. SDS has to be washed out thoroughly after boiling. Make sure the samples are boiling at 100°C. Bad isolation results are mostly caused by too low heat.] Spin down the suspension (5 min at 10,000 rpm), wash it at least twice with 1.5 ml ddH_2O and resuspend the pellet in 1 ml ddH_2O. Put the sample to a sonifier waterbath for 30 minutes. Add 500 µl of solution B (15 µg/ml DNase and 60 µg/ml RNase in 0.1 M TRIS/HCl, pH 6.8) and incubate for 60 minutes at 37°C in a shaker. Add 500 µl of solution C (50 µg/ml trypsin in ddH_2O) and incubate for additional 60 minutes at the same conditions. To inactivate the enzymes boil the suspension for 3 minutes at 100°C in a heating block, then spin the sample down (5 min at 10,000 rpm) and wash it once with 1 ml ddH_2O. To release WTA resuspend the pellet in 500 µl of 1 M HCl (ready-to-use solution from Applichem) and incubate for 4 h at 37°C in a shaker. Spin down the suspension (5 min at 10,000 rpm) and wash with ddH_2O until the pH is 5–6. Afterwards, resuspend the pellet in 100–250 µl digestion buffer (12.5 mM sodium dihydrogen-phosphate, pH 5.5) to an OD_{578} of 3.0 and add 1/10 volume of mutanolysin solution (5.000 U/ml of mutanolysin in ddH_2O). [Δ CRITICAL STEP 2: If OD_{578} is too high the sample is too concentrated. Therefore, the digestion with mutanolysin might be disturbed. Measurement of OD is tricky, because peptidoglycan sinks to the bottom. Mix the suspension carefully with a pipette and measure OD rapidly.] Then incubate the sample for 16 h at 37°C (150 rpm shaking). Inactivate mutanolysin by boiling (100°C) for 3 min. Spin the sample down (5 min at 10,000 rpm) and use the supernatant. Before applying the sample to the UPLC system, MurNAc has to be reduced to NAc-muraminitol. Therefore, add 50 µl of reduction solution (10 mg/ml sodium borohydrate in 0.5 M borax in ddH_2O at pH 9.0; both reagents were purchased from Merck) and incubate the sample for 20 minutes at room temperature. Stop the reaction with 10 µl phosphoric acid (98%). The resulting pH must be between 2 and 3. Then analyze the sample by UPLC/MS or HPLC/MS.

Minipreparation in 96 well plate for UPLC/MS analysis. This protocol gives just enough material for one sample for UPLC/MS analysis. For different growth parameters, use a divisible 96 well plate. Use a multichannel pipette for all resuspension steps. Always cover the samples properly with a foil (Greiner) or lid to avoid evaporation or mixing of samples!

Always use a 96 well-plate with U-shaped bottom (Greiner). Spin down 200 µl of the culture for 10 min at 4,700 rpm in a plate centrifuge. Resuspend each pellet in 200 µl solution A (1 M sodium chloride) and boil the suspension for 30 minutes at 100°C in a heating block. [Δ CRITICAL STEP 1: Sometimes, NaCl treatment is not sufficient for peptidoglycan isolation from the cells. Use 0.25% SDS solution in 0.1 M Tris/HCl (pH 6.8) instead. SDS has to be washed out thoroughly after boiling.] The plate must be covered with foil. Make sure the samples are boiling at 100°C. Bad isolation results are mostly caused by too low heat. Spin down the suspension (10 min at 4,700 rpm with foil coverage or lid) and wash at least twice with 200 µl ddH_2O. Afterwards, resuspend each pellet in 150 µl ddH_2O. Close the wells carefully with a foil coverage (NOT lid), so that no water can ingress to the sample. Place the plate to a sonifier waterbath for 30 minutes (plate floats). Spin down the suspension (10 min at 4,700 rpm) and resuspend each pellet in 100 µl of solution B (15 µg/ml DNase and 60 µg/ml RNase in 0.1 M TRIS/HCl, pH 6.8). Properly close plate with foil coverage and incubate for 60 minutes at 37°C in a shaker at 150 rpm. It is better to place samples at the edge of the shaker than in the middle. Add 100 µl of solution C (50 µg/ml trypsin in ddH_2O), properly close plate with foil coverage and incubate for another 60 minutes at 37°C in a shaker at 150 rpm. To inactivate the enzymes boil the suspension for 5 minutes at 100°C. Spin down the suspension (10 min at 4,700 rpm) and wash each pellet once with 200 µl ddH_2O. To release WTA resuspend each pellet in 200 µl of 1 M HCl (ready-to-use solution from Applichem) and incubate for 4 h at 37°C in a shaker (150 rpm, with foil coverage). Spin down the suspension (10 min at 4,700 rpm) and wash pellets with ddH_2O until the pH is 5–6. Resuspend each pellet in 50 µl digestion buffer (12.5 mM sodium dihydrogen-phosphate, pH 5.5) and add 5 µl mutanolysin solution (5.000 U/ml of mutanolysin in ddH_2O). Incubate the well plate for 16 h at 37°C (at 150 rpm, with foil coverage). Boil the samples on the heating block (100°C) for 5 minutes to inactivate mutanolysin. Afterwards spin the plate down (10 min at 4,700 rpm) and use the supernatant. Before applying the samples to the UPLC system, MurNAc has to be reduced to NAc-muraminitol. Add 10 µl of the reduction solution (10 mg/ml sodium borohydrate in 0.5 M borax in ddH_2O at pH 9.0; both reagents were purchased from Merck) to each sample and incubate for 20 min at room temperature. [Δ CRITICAL STEP 2: The reduction solution contains a lot of bubbles. Transfer of the exact volume of 10 µl reduction solution is not possible. Set your pipette to a volume of 100 µl and add 1 drop to each sample. This accords to the needed volume.] Stop the reaction with 5 µl phosphoric acid (50%). The pH must be between 2 and 3 (add phosphoric acid very slow and carefully!). Then analyze the samples by UPLC or UPLC/MS.

UPLC/MS ANALYSIS. An Acquity UPLC was coupled to a SynaptG2 mass spectrometer (both Waters). We used a C18 CSH 130Å, 1.7 µm, 2.1 mm × 100 mm column with the respective guard column (C18 CSH 130Å, 1.7 µm, 2.1 mm × 5 mm) available at Waters. As MS standard L-Enk was used.

The column temperature was 52°C and the injection volume was 10 µl with no loop overfill. Muropeptides were detected at 210 nm (DAD) or by MS. The MS was set to positive ESI mode with a scan range from 50–2,000. The capillary voltage was 3 kV. The sampling cone was set to 30 and the extraction cone to 3.0. Source temperature was set to 120°C and desolvation temperature to 450°C. The flow of the cone gas was 10 l/h and of the desolvation gas 800 l/h.

For muropeptide separation by UPLC/MS we used solvent A (0.1% TFA in 5% methanol; for Gram-negatives omit methanol) and solvent B (0.1% TFA in 30% methanol). TFA (trifluoroacetic acid) was bought from Carl Roth, methanol (UV grade) from Sigma-Aldrich. We applied a flow rate of 0.176 ml/min starting with 100% solvent A for 1 min. Afterwards, a linear gradient was run in 59 min to 100% solvent B. The post run was 5 min with 100% solvent B and additional 10 min with 100% solvent A for re-equilibration. [Δ CRITICAL STEP: A long and intensive equilibration of the column and an exact column temperature is very important.

Wash the column 30 min with methanol, 30 min with ddH_2O water, 30 min with solvent B, and 30 min with solvent A to a steady baseline. Degassed solvents should be self-evident.]

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Author contributions

D.K., M.S., D.D.D., Conception and design, Acquisition of data, Analysis and interpretation of data; U.B., Conception and design, Interpretation of data, Writing the article.

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