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

Ute Bertsche (ute.bertsche@uni-tuebingen.de)
IMIT - Microbial Genetics - University of Tuebingen
Daniel Kühner
IMIT - Microbial Genetics - University of Tuebingen
Mark Stahl
ZMBP - Central Facilities - University of Tuebingen
Dogan D. Demircioglu
IMIT - Microbial Genetics - University of Tuebingen

Method Article

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Abstract

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.

Introduction

The bacterial cell wall is mainly composed of peptidoglycan \(PGN). Since its first isolation in 1951 by Salton and Horne \(1) PGN composition has been intensively studied with various methods. The original determination of the glycan and peptide composition was performed by paper chromatography \(2). 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 _Escherichia 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. 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 \(6). As an example

for PGN isolation by this new method, UPLC analysis and MS-mapping are presented in "Fig. 1":http://www.nature.com/protocolexchange/system/uploads/3367/original/Figure-1_Bertsche_.tif?1417688252 and "Fig. 2":http://www.nature.com/protocolexchange/system/uploads/3369/original/Figure-2_Bertsche_.tif?1417688768. For Gram-positives we used _Staphylococcus aureus_ \(_S. aureus_) SA1137 and for Gram-negatives _Escherichia coli_ \(_E. coli_) Nissle 1917 \(8). The complete results of the muropeptide composition of _S. aureus_ are given in "Tab. 1":http://www.nature.com/protocolexchange/system/uploads/3371/original/Table_1.pdf?1417689409 and in "Tab. 2":http://www.nature.com/protocolexchange/system/uploads/3373/original/Table_2.pdf?1417689597 for _E. coli_.

Reagents

CHEMICALS • Borax \(Merck) • DNase \(Sigma-Aldrich) • ddH_2O \(deionized, HPLC grade >18MΩ) \(MilliQ) • Hydrochloric acid \(Applichem) • HPLC solvents A and B \(Table 3) • L-Enk \(Sigma-Aldrich) • Methanol UV grade \(Sigma-Aldrich) • Mutanolysin \(Sigma-Aldrich) • Phosphoric acid 98% \(Sigma-Aldrich) • RNase \(Sigma-Aldrich) • Sodium borohydride \(Merck) • Sodium chloride \(Carl Roth) • Sodium dodecylsulfate \(Sigma-Aldrich) • Sodium dihydrogenphosphate \(Sigma-Aldrich) • Trifluoroacetic acid \(Carl Roth) • Trizma base \(Sigma-Aldrich) • Trypsin \(Sigma-Aldrich) _BACTERIAL STRAINS AND MEDIA_ _S. aureus_ SA113 \(7) _E. coli_ Nissle 1917 \(8) Basic medium \(BM) for _S. aureus_ SA113: • Soy Peptone 10 g \(Plato) • Yeast Extract 5 g \(Deutsche Hefewerke) • NaCl 5 g \(Carl-Roth) • Glucose 1 g \(Carl Roth) • K_2HPO_4 1 g \(Applichem) Add deionized water to a final volume of 1 liter and adjust pH to 7.2. LB medium for _E. coli_ Nissle 1917: • Peptone 10 g \(Plato) • Yeast Extract 5 g \(Deutsche Hefewerke) • NaCl 5 g \(Carl Roth) Add deionized water to a final volume of 1 liter and adjust pH to 7.2.

Equipment

• Eppendorf tubes \(2 ml, Eppendorf) • 96 well-plate with U-shaped bottom \(Greiner) • Foil coverage \(Greiner) • Heating block \(Liebisch) • Incubator with shaking function \(Infors) • On desk centrifuge \(Eppendorf) with rotor for Eppendorf cups or 96 well plate • Vortexer \(Vortex) • Waterbath with sonification function \(Sonorex) • UPLC Acquity H-class coupled to a SynaptG2 mass spectrometer \(Waters) • UPLC column: CSH C18, 130Å, 1.7 µm, 2.1 mm x 100 mm \(Waters) • Guard column: C18 CSH 130Å, 1.7 µm, 2.1 mm x 5 mm \(Waters)

Procedure

The presented step-to-step protocols are applicable to Gram-positives as well as to Gram-negatives. All solutions and buffers are given in "Tab. 3":http://www.nature.com/protocolexchange/system/uploads/3375/original/Table_3.pdf?1417694067. For different specifications there are two protocols available. The protocol for midipreparation \("Tab. 4":http://www.nature.com/protocolexchange/system/uploads/3377/original/Table_4.pdf?1417694125)

results in sample amounts suitable for several analyses. From 2 ml of culture of OD578 ≈ 10 about 300 µl purified PGN are gained. The minipreparation protocol \("Tab. 5":http://www.nature.com/protocolexchange/system/uploads/3389/original/Table_5.pdf?1418025483) can be performed in a 96 well plate and 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. _UPLC/MS ANALYSIS_ UPLC \(Waters) Acquity H-class coupled to a SynaptG2 mass spectrometer Solvent A: 0.1% TFA in 5% methanol \(for Gram-negatives omit methanol) Solvent B: 0.1% TFA in 30% methanol Column: C18 CSH 130Å, 1.7 µm, 2.1 mm x 100 mm Guard column: C18 CSH 130Å, 1.7 µm, 2.1 mm x 5 mm Column temperature: 52°C Injection volume: 10 µl Loop: no loop overfill Detection: 210 nm \(DAD) Mass spectrometer: SynaptG2 Mode: positive ESI mode with a scan range from 50 – 2,000 Capillary Voltage: 3 kV Sampling Cone: 30 Extraction Cone: 3.0 Source Temperature: 120 °C Desolvation Temperature: 450 °C Cone Gas: 10 l/h Desolvation Gas: 800 l/h MS standard: L-Enk UPLC gradient conditions are given in "Tab. 6":http://www.nature.com/protocolexchange/system/uploads/3385/original/Table_6.pdf?1417694618.

Troubleshooting

For troubleshooting please refer to "Tab. 7":http://www.nature.com/protocolexchange/system/uploads/3387/original/Table_7.pdf?1417694650 for both protocols.

Anticipated Results

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 \("Fig. 1":http://www.nature.com/protocolexchange/system/uploads/3367/original/Figure-1_Bertsche_.tif?1417688252). 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 "Tab. 1":http://www.nature.com/protocolexchange/system/uploads/3371/original/Table_1.pdf?1417689409 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 \(4,9). 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 \(peaks 5, 11, 15, 16, 17 and 18) contained the expected muropeptides that had already been determined before \(4,10). We mapped the whole PGN of _S. aureus_ SA113 up to the hexameric cross-linked muropeptide. The complete results of

our PGN mapping of _S. aureus_ SA113 is given in "Tab. 1":http://www.nature.com/protocolexchange/system/uploads/3371/original/Table_1.pdf?1417689409. 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 were detected. _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":http://www.nature.com/protocolexchange/system/uploads/3369/original/Figure-2_Bertsche_.tif?1417688768). 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). The complete analysis is given in "Tab. 2":http://www.nature.com/protocolexchange/system/uploads/3373/original/Table_2.pdf?1417689597. 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.

References

1 Salton, M. R. J., Horne, R. W. Studies of the bacterial cell wall. 2. Methods of preparation and some properties of cell walls. Biochimica et Biophysica Acta 7, 177-197 \(1951). 2 Schleifer, K. H. & Kandler, O. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36, 407-477. \(1972). 3 Glauner, B. Separation and quantification of muropeptides with high-performance liquid chromatography. Anal Biochem 172, 451-464. \(1988). 4 de Jonge, B. L., Chang, Y. S., Gage, D. & Tomasz, A. Peptidoglycan composition of a highly methicillin-resistant _Staphylococcus aureus_ strain. The role of penicillin binding protein 2A. J Biol Chem 267, 11248-11254 \(1992). 5 Desmarais, S. M., Cava, F., de Pedro, M. A. & Huang, K. C. Isolation and Preparation of Bacterial Cell Walls for Compositional Analysis by Ultra Performance Liquid Chromatography. JoVE, e51183, doi:doi:10.3791/51183 \(2014). 6 Müller-Anstett, M. A. et al. Staphylococcal peptidoglycan co-localizes with Nod2 and TLR2 and activates innate immune response via both receptors in primary murine keratinocytes. PLoS One 5, e13153, doi:10.1371/journal.pone.0013153 \(2010). 7 Iordanescu, S. Host controlled restriction mutants of _Staphylococcus aureus_. Archives roumaines de pathologie experimentales et de microbiologie 34, 55-58 \(1975). 8 Jacobi, C. A. & Malfertheiner, P. _Escherichia coli_ Nissle 1917 \(Mutaflor): new insights into an old probiotic bacterium. Digestive diseases \(Basel, Switzerland) 29, 600-607, doi:10.1159/000333307 \(2011). 9 de Jonge, B. L., Chang, Y. S., Gage, D. & Tomasz, A. Peptidoglycan composition in heterogeneous Tn551 mutants of a methicillin-resistant _Staphylococcus aureus_ strain. J Biol Chem 267, 11255-11259 \(1992). 10 Boneca, I. G., Xu, N., Gage, D. A., de Jonge, B. L. & Tomasz, A. Structural characterization of an abnormally cross-linked muropeptide dimer that is accumulated in the

peptidoglycan of methicillin- and cefotaxime-resistant mutants of _Staphylococcus aureus_. J Biol Chem 272, 29053-29059 \(1997).

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Figures

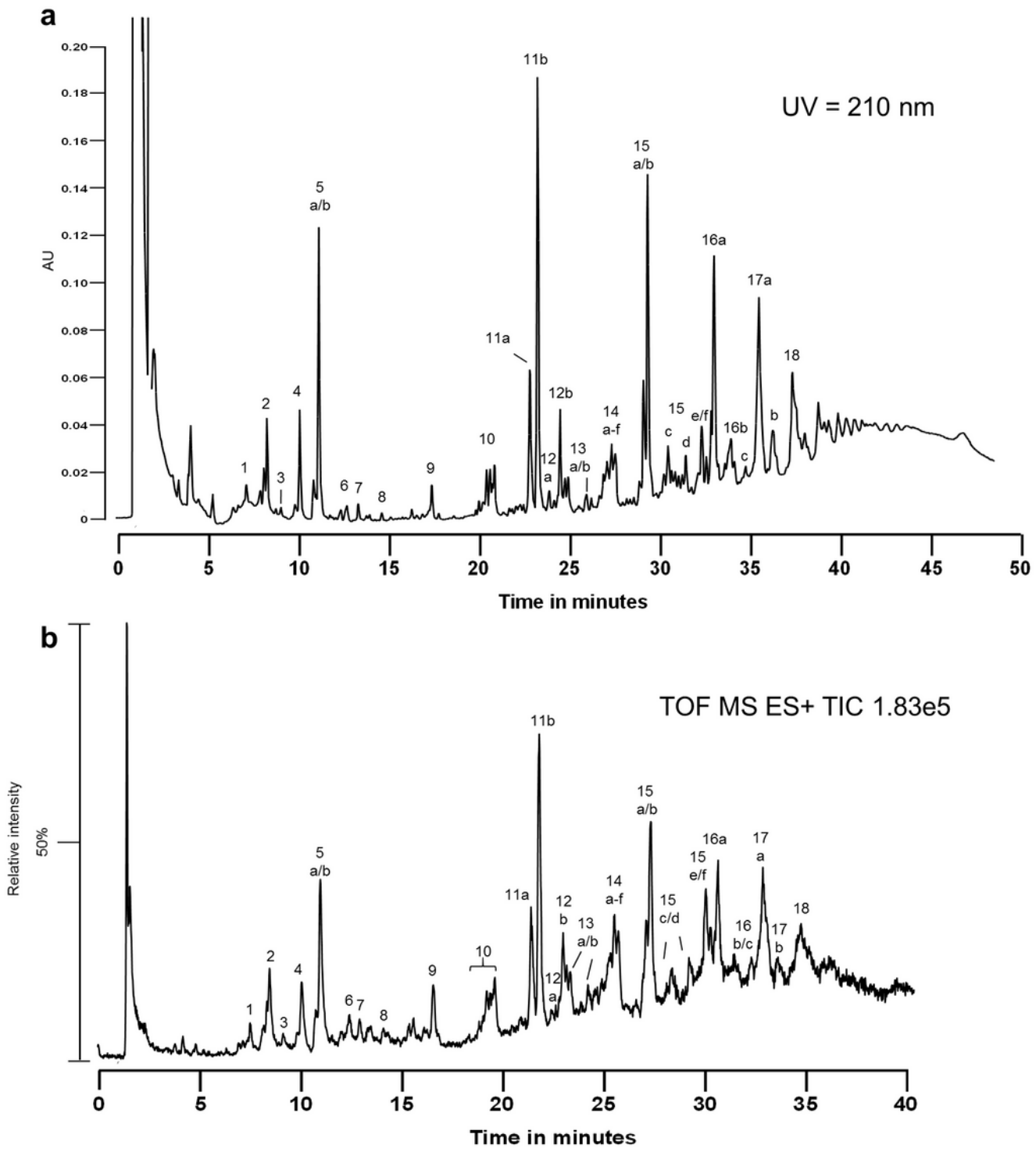

Figure 1

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 "Tab. 1":http://www.nature.com/protocolexchange/system/uploads/3371/original/Table_1.pdf?1417689409 including molecule composition and proposed sum formula.

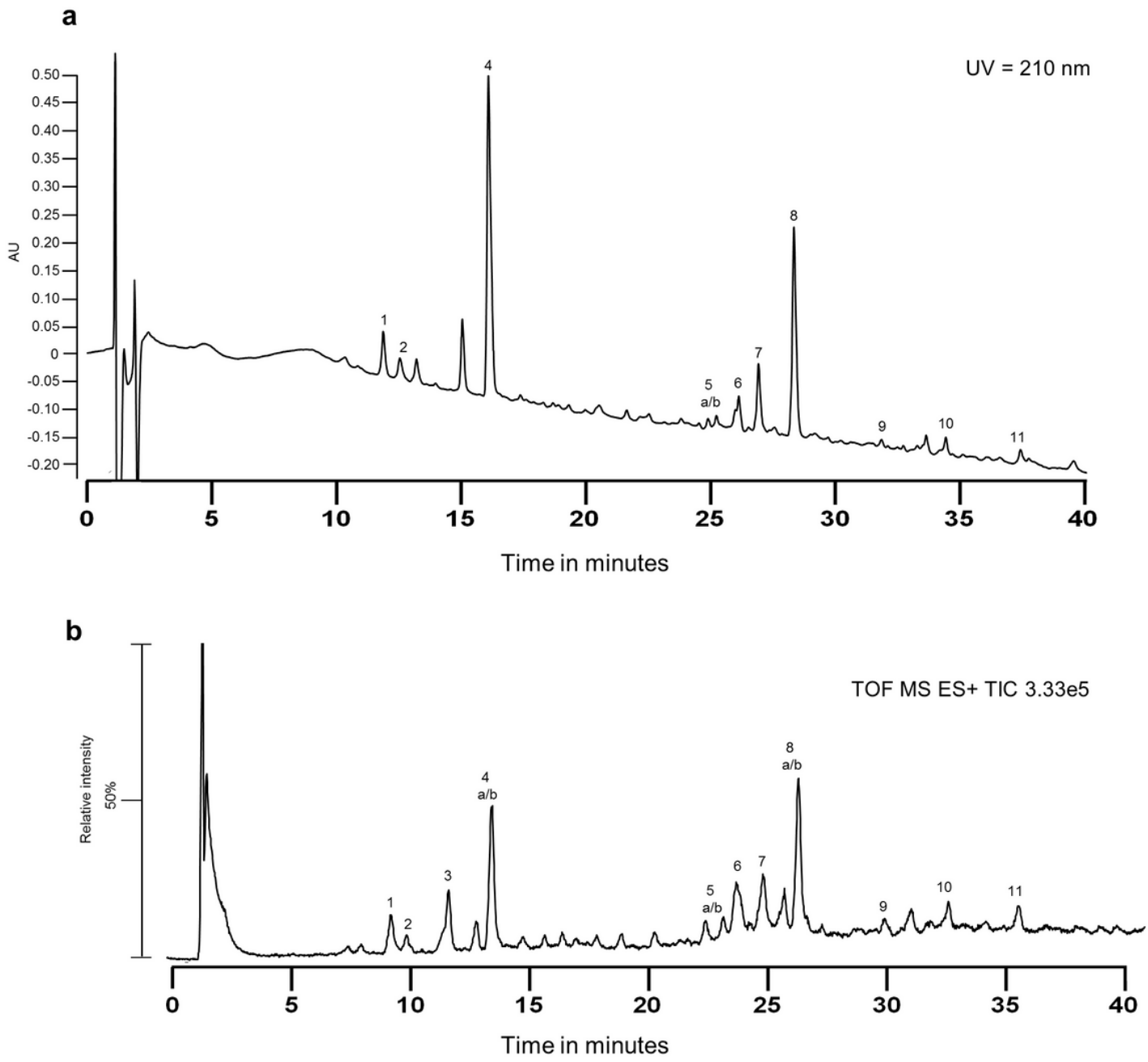

Figure 2

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 "Tab. 2":http://www.nature.com/protocolexchange/system/uploads/3373/original/Table_2.pdf?1417689597 including molecule composition and proposed sum formula.

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