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Improved high-performance liquid chromatographic separation of peptidoglycan isolated from various *Staphylococcus aureus* strains for mass spectrometric characterization

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Abstract

Reversed-phase high-performance liquid chromatography (RP-HPLC) of muropeptides, obtained by muramidase digestion of peptidoglycan in combination with amino acid analysis and plasma desorption time-of-flight mass spectrometry is today by far the best tool to analyze the fine structure of the peptidoglycans. Here we report further improvements of the RP-HPLC separation of muropeptides for analyzing the peptidoglycans of various methicillin-resistant strains of *Staphylococcus aureus*, with emphasis on a more detailed characterization of the interpeptide bridge of the peptidoglycans of this species.

Keywords: *Staphylococcus aureus*; Peptidoglycan

1. Introduction

The dramatic increase of multiresistant *Staphylococcus aureus* infections (MRSA) has become an important infection control problem worldwide [1–4]. The expression of resistance against all β-lactams is based on the additional *mecA* gene and several *fem* factors, which are natural constituents of the staphylococcal genome. The *mecA* gene codes for a low-affinity PBP (penicillin binding protein), called PBP2′ or PBP2a. The PBPs are involved in the crosslinking of the bacterial cell wall. This PBP2′ is still functioning in cell wall synthesis at high β-lactam concentrations, which will stop the action of all other PBPs [5]. Preceding publications showed that the *fem* factors A and B, the most important ones with respect to full expression of resistance, are involved in the biosynthesis of the staphylococcal interpeptide bridge [6–10]. These results lead to the idea that the biosynthesis of the interpeptide bridge is a novel target for staphylococcal-specific antibiotics [9,11]. The results gained for the structure of the peptidoglycan under investigation could be obtained by RP-HPLC of muramidase-digested peptidoglycan in combination with amino acid analysis and new mass spectrometric methods [12–14]. The improved

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method has already been applied in the following publications [9,10,15,16,18]. Here we report the methodological conditions for the successful application of a high-resolution 'off-line' combination of high-performance liquid chromatography (HPLC) with linear time-of-flight (TOF) mass spectrometry to the detailed analysis of the muropeptide composition of the cell wall peptidoglycan of staphylococci, which made it possible to unravel some of the contributions of *femA* and *femB* factors to pentaglycine interpeptide bridge synthesis.

2. Experimental

Preparation of peptidoglycan and of reduced muropeptides was performed as described elsewhere [9].

2.1. Separation of reduced muropeptides by RP-HPLC

The HPLC system consisted of an LC 1050 (Hewlett-Packard). Separation of reduced muropeptides was performed as described by Glauner [17] and de Jonge et al. [13] with several further modifications for better resolution.

2.2. Normal resolution (all muropeptide ranges)

Samples (50–100 μl) were injected on a 250×4 mm biocompatible 'CompAx-Peek' column guarded by a 14×4 mm precolumn, both filled with 3 μm ODS Hypersil (Knauer, Berlin, Germany). Column temperature was 37°C in a water bath to obtain constant temperature conditions. The flow-rate was 0.75 ml/min. The linear gradient of elution started from 5% methanol in 100 m*M* phosphate buffer, pH 2.5, containing 0.00025% sodium azide (buffer A) to 30% methanol in 100 m*M* phosphate buffer, pH 2.8 (buffer B), in 210 min. UV detection was performed at 206 nm.

2.3. High resolution (only monomer range)

Only flow-rate and methanol gradient were changed in respect to the above conditions. Flow-rate was now 0.9 ml/min; the linear gradient started from 5% methanol in 100 m*M* phosphate buffer, pH 2.5, containing 0.00025% sodium azide to 11.25% methanol in 100 m*M* phosphate buffer, pH 2.8, in 100 min.

2.4. Purification of the reduced muropeptides by RP18 HPLC for mass spectrometric analysis

Separation for mass spectrometric analysis was performed on an HP 1090 high-performance liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) using a ODS Hypersil column (Shandon Scientific, Runcorn, UK) with a particle size of 3.5 μm, pore size of 120 Å and column dimensions of 250×4 mm. The solvent gradient used was linear from 100% ultrapure water (18 $M\Omega^{-1}$ cm^{-1}) generated by an Elgastat apparatus (Elga, Bucks, UK) containing 0.05% trifluoroacetic acid (Spectro Grade, Pierce, Rockford, IL, USA) to 17.5% acetonitrile containing 0.035% trifluoroacetic acid from 5 to 65 min. The optimized flow-rate was 1 ml/min. The column temperature was maintained at 30°C and the UV absorbance detector was operated at 210 nm.

2.5. Characterization of reduced muropeptides

2.5.1. Amino acid analysis

The collected samples were lyophilized and subjected to hydrolysis (4 *M* HCl, 100°C, 18 h). Amino acid analysis of the hydrolyzed samples was performed with an LC 5001 amino acid analyzer (Biotronik, Darmstadt, Germany), using an SCEX column and a sodium citrate buffer system. The amino compounds were detected by means of the ninhydrin reagent.

2.5.2. Plasma desorption mass spectrometry

Positive and negative ion californium-252 plasma desorption (PD) mass spectra were obtained using a BioIon 20 linear time-of-flight mass spectrometer (BioIon AB, Uppsala, Sweden) under the following conditions: length of drift region, 141 mm; accelerating voltage, 17 kV for positive and 15 kV for negative ion detection; time resolution, 1 ns per channel; spectrum accumulation time, between 2×10^6 and 15×10^6 fission events for positive and 5×10^6 to 1×10^7 for negative ion mass spectra. Mass

calibration was based on H^+ and Na^+ ions for positive and on H^- and CN^- ions for negative ion PD mass spectra. All collected fractions dissolved in 2–5 μl 0.1% trifluoroacetic acid were deposited onto nitrocellulose covered targets. Afterwards, our previously described nitrocellulose sandwich technique [12], for removing still present low-molecular-weight contaminants without sample loss, was applied to obtain optimal signal-to-noise ratio for the mass spectrometric measurements.

3. Results and discussion

3.1. RP-HPLC

In order to obtain optimal resolution for the separation of the RP-HPLC of muraminidase-digested and reduced peptidoglycan, the following HPLC parameters were subject of variation: (a) column material, (b) solid phase material, (c) mobile phase and (d) temperature.

As an indication for the improvement of the separation of the muropeptides, the degree of resolution of peaks M3 and M4 was chosen. The separation of these two peaks, which represent a monomeric disaccharide pentapeptide with a triglycine or pentaglycine bridge (see Fig. 1), respectively, could not be achieved by the established method [8]. Both these structures were of great interest with respect to our research on *fem* mutants, possibly synthesizing interpeptide bridges with an altered chain length and amino acid composition.

3.1.1. Column material

The biocompatible PEEK-column, when compared to the commonly used stainless steel columns [8,13,17], showed a significant improvement of separation. The separating power of the system could be additionally optimized by combining an increased flow of the mobile phase and a shallower methanol gradient at 37°C. With this, the one main monomeric peak in the chromatograms of de Jonge et al. [8] could be separated into three main peaks, M3, M4 and M5, with some minor peaks between M3 and M4 (Fig. 1). The structure of the substance eluting in peak M5 could be identified as a monomeric disaccharide pentapeptide with a tetraglycine–monoserine bridge. Applying this improved method led to a better resolution for the whole chromatogram, e.g. splitting of peak M9 into two peaks, M9′ and M9″, corresponding to the following structures: a monomeric disaccharide pentapeptide with no amidation of the iso-D-glutamic acid and a pentaglycine bridge and monomeric disaccharide pentapeptide with a monoalanine bridge.

3.1.2. Solid phase material

Of the tested column fill materials, 3 μm Hypersil ODS revealed the highest resolution, followed by 3 μm Nucleosil ODS, 4 μm Superspher ODS, 5 μm Eurochrom ODS and 5 μm Lichrospher ODS.

3.1.3. Mobile phase

Replacement of the methanol–phosphate buffer system established by Glauner [17] with an acetonitrile–trifluoroacetic acid buffer system revealed a decrease in resolution. Adjustment of the pH of the mobile phase to higher values resulted in an alteration of the separation pattern. The mobile phase (pH 2.5) used by de Jonge et al. [13] with respect to the mobile phase (pH 4.5) used by Glauner [17] showed that all peaks were eluted faster, especially those peaks which could be attributed to structures with no amidation of the iso-D-glutamic acid of the stem peptide. However resolution was still as good as described above.

Modification of the flow from 0.2 to 0.7 ml/min revealed, in the case of the stainless steel column, an optimum resolution at 0.5 ml/min, as described before by Glauner [17]. In the case of the biocompatible PEEK column, the resolution still increased at higher flow-rates. An optimal separation in all muropeptide areas (monomer to oligomer) was found at a flow-rate of 0.75 ml/min (Fig. 2). Higher flow-rates were restricted by the high backpressure (280 bar) of the column. If separation of the monomeric muropeptides is the prime task (monomer area) a flow-rate of 0.9 ml/min appears optimal (because of the lower methanol content in the mobile phase, which causes a lower backpressure of the column) and revealed the best resolution described so far (Fig. 1).

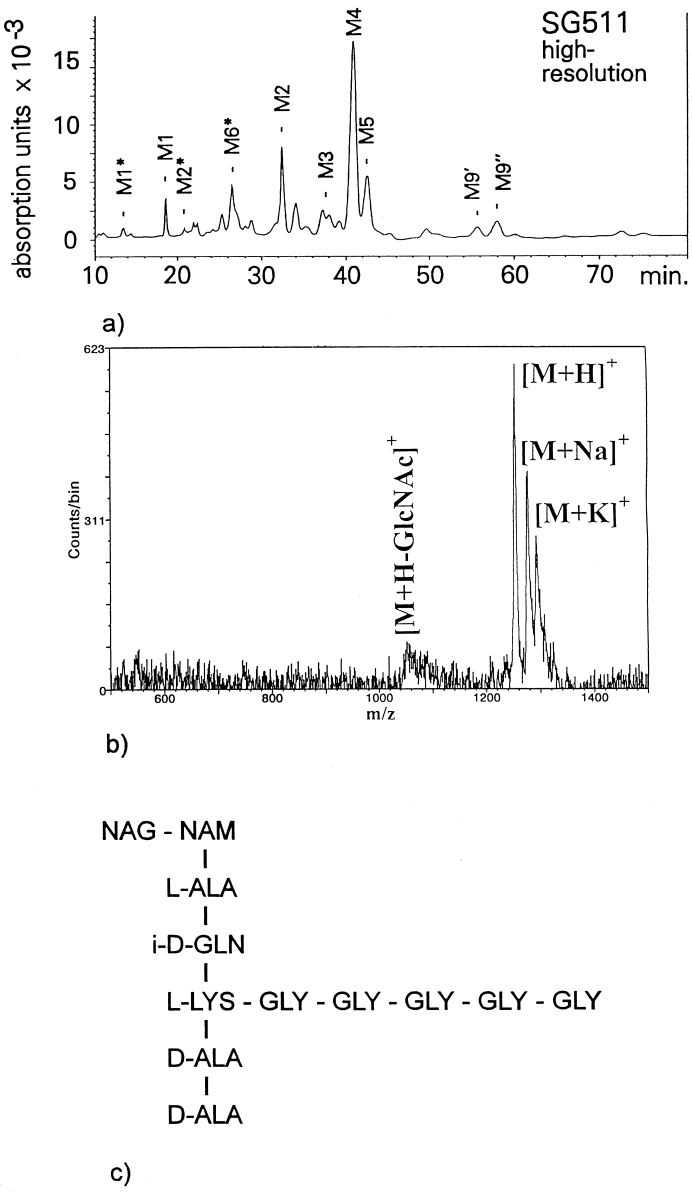

Fig. 1. High-resolution of the monomer area of the RP-HPLC of muraminidase-digested peptidoglycan of strain SG511. (b) Positive-ion PD mass spectrum of peptidoglycan monomer M4 isolated from *Staphylococcus aureus* strain SG511 after applying the nitrocellulose sandwich technique. $[MH]^+$ denotes the protonated molecular ion, the most abundant ion of the spectrum. The relative molecular mass for M4 was measured as 1252.6 (determined from the m/z values of $[MH]^+$ and $[M-H]^-$ ions). (c) Chemical structure of the main peak M4, the characteristic muropeptide monomer of *Staphylococcus aureus*.

3.1.4. Temperature

The best resolution with the new biocompatible PEEK column was found at 37°C in a water bath. Thermostatization using an air oven revealed lower reproducibility of the peak retention times. A pre-thermostatization of the mobile phase before entering the column did not improve the above-described separation.

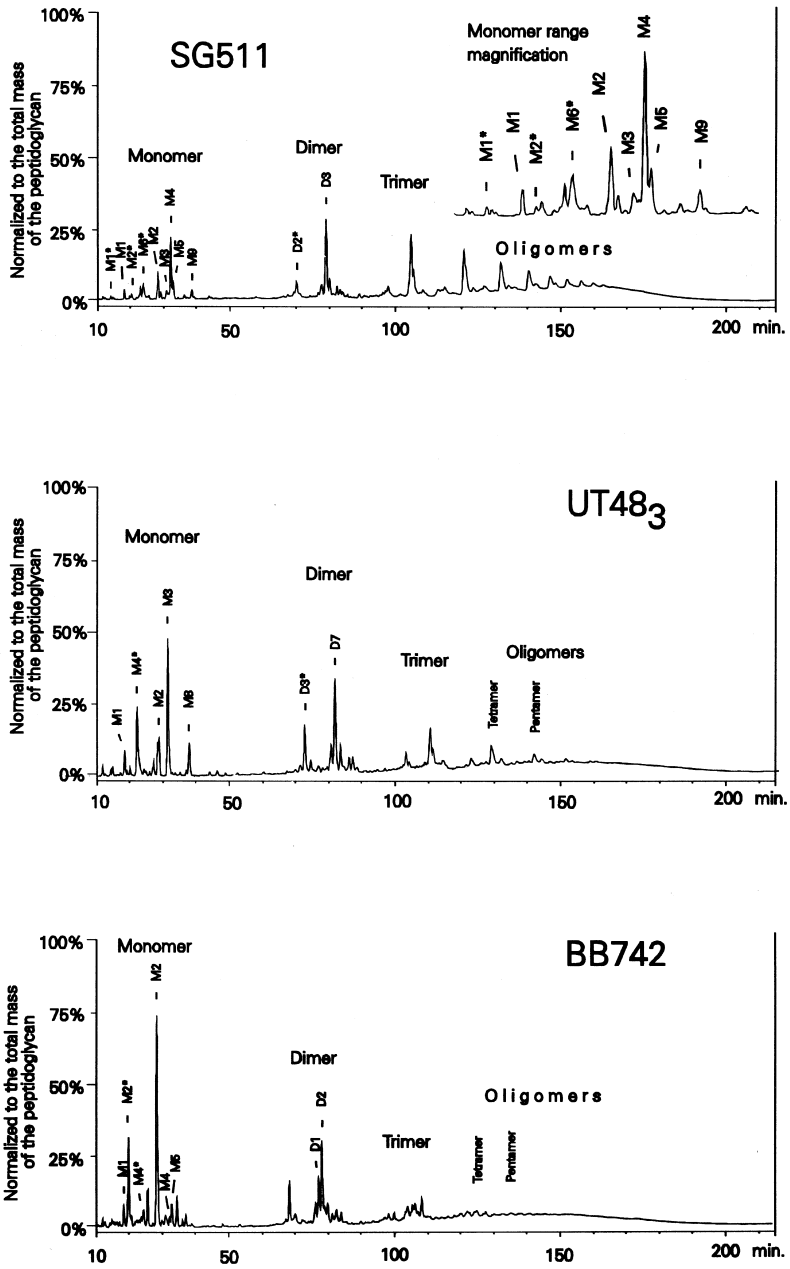

Fig. 2. Chromatograms of the RP-HPLC of muraminidase-digested peptidoglycan (normal resolution) of strains SG511, UT48$_3$ and BB742.

The analysis of peptidoglycan from strain SG511 and its *femAB* and *femB* mutants [6,7], using the described improvements of the RP-HPLC separation of muropeptides, resulted in the chromatograms shown in Figs. 1 and 2. Using amino acid analysis and Cf-252 plasma-desorption mass spectrometry (PD-MS) the peaks of the RP-HPLC could be assigned to the chemical structures listed in Tables 1–3. The assignments of the oligomers (trimer, tetramer, etc.) were achieved by gel permeation

Table 1
Calculated and measured m/z values for the protonated or sodiated and deprotonated molecular ions of major muropeptides isolated from *Staphylococcus aureus* strain SG511 Berlin

Fraction label	Type of ion	m/z		Δm[a] (Dalton)	Amino acid composition					Proposed structures
		Calculated[c]	Measured		Glx	Ser	Gly	Ser+Gly	Ala	
M1	$[M+H]^+$	969.0	969.1	+0.1	n.d.[b]	n.d.	n.d.	n.d.	n.d.	NAG-NAM-Ala-iGln-Lys-Ala-Ala
	$[M-H]^-$	967.0	966.6	−0.4						
M2	$[M+H]^+$	1026.1	−1027.0	+0.9	1	0.1.	2.0	2.1	2.7	NAG-NAM-Ala-iGln-Lys(Gly_1)-Ala-Ala
	$[M-H]^-$	—	—	—						
M3					1	0.5	2.3	2.8	2.8	NAG-NAM-Ala-iGln-Lys(Gly_3)-Ala-Ala and NAG-NAM-Ala-iGln-Lys(Gly_2Ser)-Ala-Ala
M4	$[M+H]^+$	1254.3	1253.8	−0.5	1	0.2	4.9	5.1	3.0	NAG-NAM-Ala-iGln-Lys(Gly_5)-Ala-Ala
	$[M-H]^-$	1252.3	1251.3	−1.0						
M5	$[M+H]^+$	1283.3	1282.0	−1.0	1	0.7	4.2	4.9	2.8	NAG-NAM-Ala-iGln-Lys(Gly_4Ser)-Ala-Ala
	$[M-H]^-$	1281.3	—	—						
M9					1	0.1	2.4	2.5	3.2	NAG-NAM-Ala-iGlu-Lys(Gly_5)-Ala-Ala and NAG-NAM-Ala-iGln-Lys(Ala)-Ala-Ala
M1*	$[M+H]^+$	955.0	955.4	+0.4	n.d.	n.d.	n.d.	n.d.	n.d.	NAG-NAM-Ala-iGln-Lys-Ala-Gly or NAG-NAM-Ala-iGln-Lys(Gly)-Ala
	$[M-H]^-$	953.0	953.6	+0.6						
M2*	$[M+Na]^+$	1034.1	1034.5	+0.4	n.d.	n.d.	n.d.	n.d.	n.d.	NAG-NAM-Ala-iGln-Lys(Gly_2)-Ala or NAG-NAM-Ala-iGln-Lys(Gly)-Ala-Gly
	$[M-H]^-$	—	—	—						
M6*	$[M+H]^+$	1240.3	1241.0	+0.7	1	0.5	6.2	6.7	1.8	NAG-NAM-Ala-iGln-Lys(Gly_5)-Ala-Gly or NAG-NAM-Ala-iGln-Lys(Gly_4)-Ala-Gly_2
	$[M-H]^-$	1238.3	1237.9	−0.4						NAG-NAM-Ala-iGln-Lys(Gly_4Ser)-Ala-Gly or NAG-NAM-Ala-iGln-Lys(Gly_3Ser)-Ala-Gly_2
	$[M+H]^+$	—	—	—						
	$[M-H]^-$	1268.3	1269.1	+0.8						NAG-NAM-Ala-iGln-Lys(Gly_5) -Ala-Ala | NAG-NAM-Ala-iGln-Lys(Gly_5) -Ala
D3	$[M+H]^+$	2418.5	2417.9	−0.6	1	0.4	4.1	4.5	2.4	
	$[M-H]^-$	2416.5	2413.9	−2.6						NAG-NAM-Ala-iGln-Lys(Gly_5) -Ala-Gly | NAG-NAM-Ala-iGln-Lys(Gly_5) -Ala
D2*	$[M+H]^+$	2404.5	2403.1	−1.4	1	0.4	4.5	4.9	1.7	
	$[M-H]^-$	2402.4	2339.8	−2.6						

[a]Mass difference between measured and calculated protonated, sodiated or deprotonated molecular mass values.
[b]n.d., not determined.
[c]Glx stands for the glutamic acid after acid hydrolysis. MS data are calculated by mass values of Gln.

Table 2
Calculated and measured m/z values for the protonated or sodiated and deprotonated molecular ions of major muropeptides isolated from *Staphylococcus aureus* strain UT48$_3$

Fraction label	Type of ion	m/z		Δm[a] (Dalton)	Amino acid composition					Proposed structures
		Calculated[c]	Measured		Glx	Ser	Gly	Ser+Gly	Ala	
M1	$[M+H]^+$	969.0	968.0	−1.0	n.d.[b]	n.d.	n.d.	n.d.	n.d.	NAG-NAM-Ala-iGln-Lys-Ala-Ala
	$[M-H]^-$	967.0	—	—						
M2	$[M+Na]^+$	1048.1	1049.1	+1.0	1	0.1	2.0	2.1	2.7	NAG-NAM-Ala-iGln-Lys(Gly_1)-Ala-Ala
	$[M-H]^-$	1024.1	—	—						NAG-NAM-Ala-iGlu-Lys(Gly_2)-Ala-Gly
	$[M+Na]^+$	1092.1	1092.7	+0.6						
	$[M-H]^-$	—	—	—						
M3	$[M+H]^+$	1140.2	1139.2	−1.0	1	0.2	2.8	3.0	2.9	NAG-NAM-Ala-iGln-Lys(Gly_3)-Ala-Ala
	$[M-H]^-$	1138.2	1137.7	−0.5						
M8	$[M+Na]^+$	1164.2	1165.5	+1.3	n.d.	n.d.	n.d.	n.d.	n.d.	NAG-NAM-Ala-iGlu-Lys(Gly_3)-Ala-Ala
	$[M-H]^-$	—	—	—						
M1*	$[M+H]^+$	955.0	955.9	+0.9	n.d.	n.d.	n.d.	n.d.	n.d.	NAG-NAM-Ala-iGln-Lys(Gly)-Ala or NAG-NAM-Ala-iGln-Lys-Ala-Gly
	$[M-H]^-$	—	—	—						
M4*	$[M+H]^+$ $[M-H]^-$	1126.2	1125.2	−1.0	1	—	2.9.	2.9	1.7	NAG-NAM-Ala-iGln-Lys(Gly_3)-Ala-Gly or NAG-NAM-Ala-iGln-Lys(Gly_2)-Ala-Gly_2
		1124.2	1123.4	−0.8						NAG-NAM-Ala-iGln-Lys(Gly_3) -Ala-Ala
D7	$[M+H]^+$ $[M-H]^-$	2190.3	2189.5	−0.8	1	0.4	4.1	4.5	2.4	NAG-NAM-Ala-iGln-Lys(Gly_3) -Ala
		2188.3	—	—						NAG-NAM-Ala-iGln-Lys(Gly_3) -Ala-Gly
D3*	$[M+H]^+$	2176.2	2175.7	−0.6	1	—	3.0	3.0	2.1	NAG-NAM-Ala-iGln-Lys(Gly_3) -Ala
	$[M-H]^-$	2174.2	—	—						NAG-NAM-Ala-iGln-Lys(Gly_2Ser) -Ala-Gly
	$[M+H]^+$	2206.3	2205.2	−1.3						NAG-NAM-Ala-iGln-Lys(Gly_3) -Ala
	$[M-H]^-$	2204.3	—	—						

[a]Mass difference between measured and calculated protonated, sodiated or deprotonated molecular mass values.
[b]n.d., not determined.
[c]Glx stands for the glutamic acid after acid hydrolysis. MS data are calculated by mass values of Gln.

Table 3
Calculated and measured m/z values for the protonated or sodiated and deprotonated molecular ions of major muropeptides isolated from *Staphylococcus aureus* strain BB742

Fraction label	Type of ion	m/z		Δm[a] (Dalton)	Amino acid composition					Proposed structures
		Calculated[c]	Measured		Glx	Ser	Gly	Ser+Gly	Ala	
M1	$[M+H]^+$	969.0	969.4	+0.4	n.d.[b]	n.d.	n.d.	n.d.	n.d.	NAG-NAM-Ala-iGln-Lys-Ala-Ala
	$[M-H]^-$	967.0	—	—						
M2	$[M+H]^+$	1026.1	1026.0	+0.1	1	—	1.3	1.3	3.2	NAG-NAM-Ala-iGln-Lys(Gly_1)-Ala-Ala
	$[M-H]^-$	1024.1	1023.0	−1.1						
M4	$[M+H]^+$	1254.3	1254.5	+0.2	n.d.	n.d.	n.d.	n.d.	n.d.	NAG-NAM-Ala-iGln-Lys(Gly_5)-Ala-Ala
	$[M-H]^-$	1252.3	—	—						
M5	$[M+H]+$	1283.3	1284.2	+0.9	1	0.9	3.4	4.3	3.1	NAG-NAM-Ala-iGln-Lys(Gly_4Ser)-Ala-Ala
	$[M-H]^-$	1281.3	—	—						
M2*	$[M+H]^+$	1012.1	1012.3	+0.2	1	—	2.0	2.0	2.1	NAG-NAM-Ala-iGln-Lys(Gly_1)-Ala-Gly
	$[M-H]^-$	1010.1	1009.1	−1.0						
M4*	$[M+H]^+$	1099.2	1099.7	+0.5	1	0.5	2.6	3.1	2.0	NAG-NAM-Ala-iGln-Lys(GlySer)-Ala-Gly
	$[M-H]^-$	1097.2	—	—						NAG-NAM-Ala-iGln-Lys(Gly_4Ser) -Ala-Ala | NAG-NAM-Ala-iGln-Lys(Gly) -Ala
D1	$[M+H]^+$	2220.3	2220.3	0	1	0.5	2.8	3.2	2.4	
	$[M-H]^-$	2218.3	—	—						
				—						NAG-NAM-Ala-iGln-Lys(Gly) -Ala-Ala | NAG-NAM-Ala-iGln-Lys(Gly) -Ala
D2	$[M+H]^+$	1962.1	1962.9	+0.8	1	0.2	1.8	2.0	2.6	
	$[M-H]^-$	1960.0	—	—						

[a]Mass difference between measured and calculated protonated, sodiated or deprotonated molecular mass values.
[b]n.d., not determined.
[c]Glx stands for the glutamic acid after acid hydrolysis. MS data are calculated by mass values of Gln.

[7,8]. The structures of substances which eluted in peaks M9′ and M9″ were obtained by amino acid analysis with the precolumn derivatization system 'OPA–*N*-acetyl-L-cysteine' (data not shown) [18]. This amino acid analysis showed a rather high sensitivity and could resolve all peptidoglycan amino acids in their D- and L-enantiomers [19]. Supplementary, the substance of peak M9′ was characterized by standards of *Staphylococcus aureus* strains, which are known to demonstrate an unusually high content of D-Glu instead of D-Gln in peptidoglycan composition [15,18].

3.2. Mass spectrometry

To analyze the chemical structure of the monomeric and dimeric peptidoglycans by PDMS, the individual peaks of the RP18 HPLC (Fig. 2) were collected, lyophilized and afterwards rechromatographed by applying the optimized water–acetonitrile–TFA gradient RP18 HPLC system to remove the salt contaminants from monomeric and dimeric peptidoglycan compounds. Positive and sometimes negative ion PD mass spectra for all fractions could be obtained. The dominant peaks in the positive ion mode using the nitrocellulose sandwich technique [14] were protonated molecular ions, $[M+H]^+$, accompanied by less abundant sodium or potassium adduct ions (see Fig. 1). The negative ion spectra exhibited mainly deprotonated molecular ions, $[M-H]^-$. Sometimes fragment ions in the positive ion mode (see Fig. 1) were detected corresponding to $[M+H-GlcNAc]^+$ or $[M+Na-GlcNAc]^+$. Tables 1–3 contain the detailed mass spectrometric data and amino acid data resulting in structural proposals for the main monomeric and dimeric peptidoglycan compounds of the three *Staphylococcus aureus* strains.

For the parent strain SG511, Table 1 shows that compounds containing glycine bridges shorter than five glycines were found only in minor amounts. Additional peaks of significant quantities could be assigned to structures originating after processing with a peptidoglycan endopeptidase. Table 2 exhibits only minor peaks for the *femB* mutant UT483, representing compounds with glycine bridges smaller than three glycine molecules. In the monomer region, one significant peak with three glycine molecules was found. Compounds with five glycines per bridge were not found. For the *femAB* mutant BB742 no compound could be detected in the monomer region, representing considerable amounts of glycine bridges containing more than one glycine (see Table 3).

In conclusion, the structure of the interpeptide bridge of staphylococcal peptidoglycan could be well determined by an improved resolution of the RP-HPLC of muraminidase-digested and reduced peptidoglycan. In this way, modifications of the structure of the interpeptide bridge in different *Staphylococcus aureus* strains [18], as well as alterations caused by antibiotics [13,18] or genetic manipulation (e.g. *fem* mutants), could be easily analyzed.

4. Notation

NAG or GluNAc	*N*-acetylglucosamine
NAM or MurNAc	*N*-acetylmuramic acid
L-Ala	L-alanine
i-D-Gln	iso-D-glutamine
L-Lys	L-lysine
D-Ala	D-alanine
Gly	glycine
ODS	octadecylsilan
CompAx PEEK	compatible axial lockable polyetherether-ketone
PBP	penicillin-binding protein
RP-HPLC	reversed-phase high-performance liquid chromatography
SCEX	strong cation exchange

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