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Structural elucidation of the extracellular and cell-wall teichoic acids of *Staphylococcus epidermidis* RP62A, a reference biofilm-positive strain

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Abstract—The ability to adhere to artificial surfaces and form biofilms is considered as a virulence factor of *Staphylococcus epidermidis*, one of the major causes of nocosomial infections, especially those related to implanted medical devices. Cell-wall teichoic acid is known to play an important role in biofilm formation of staphylococci. The structure of the cell wall and extracellular teichoic acids of *S. epidermidis* RP62A, a reference biofilm-positive strain, was studied by NMR spectroscopy and capillary electrophoresis–mass spectrometry. Their structures were found to be a (1→3)-linked poly(glycerol phosphate), substituted at the 2-position of glycerol residues with α-Glc, α-GlcNAc, D-Ala and α-Glc6Ala. D-Alanyl acylation of a sugar hydroxyl group seems to be a novel structural feature of teichoic acids from staphylococci.
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1. Introduction

Staphylococcus aureus and coagulase-negative staphylococci (CoNS), with *Staphylococcus epidermidis* as a leading species, have emerged as the most frequently isolated pathogens in nocosomial sepsis[1] and cause more infections in medical devices than any other group of microorganism.

S. epidermidis, the predominant inhabitant of skin and mucous membranes, is now known to be an opportunistic pathogen, causing infections in immunocompromised hosts or patients with implanted medical devices, such as intravascular and peritoneal dialysis catheters, prosthetic heart valves and orthopaedic prostheses. A characteristic feature of infections around prostheses and other medical devices is the ability of microorganisms to adhere and colonise surfaces of biomaterials in multilayered clusters, called biofilms, in which structured cell communities are enclosed in self-produced polymeric matrix, an amorphous slimy material, loosely bound to staphylococcal cells. This ability to form biofilms is believed to make the organisms more resistant to antibiotics and host defences.[2]

S. epidermidis strain RP62A (ATCC 35984) is considered as a reference biofilm-positive strain. According to recent studies, cell-wall teichoic acid (CW TA) significantly enhances adhesion of *S. epidermidis* to fibronectin coated surfaces. This observation may suggest a potential role of CW TA as a bridging molecule between microorganisms and immobilised fibronectin in early steps of *S. epidermidis* pathogenesis,[3] the primary attachment of cells to an artificial surface.[4]

D-Alanine esterification decreases the total negative charge of teichoic acid and thus modulates the negative charge of bacterial cell wall. The importance of D-alanine substituents of *S. aureus* teichoic acids in bacterial pathogenesis was recently intensively investigated.[5–7] Peschel et al. have described a mutant of *S. aureus*

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biofilm-positive strain Sa133, lacking D-alanine esters in its TA, since the *dlt* operon, responsible for the D-alanine transfer into TA, was disrupted. The mutant was sensitive to cationic pore-forming antimicrobial peptides,[5] had a reduced rate of autolysis, and an increased susceptibility to glycopeptide antibiotics.[6] The mutant also exhibited more than 50% reduced level of initial binding to plastic surfaces, indicating that the initial step of biofilm formation was affected by the loss of the D-alanine esters.[7]

A knowledge of the chemical structure of the CW TA of staphylococci and in particular the D-alanine substitution is essential for clear understanding of bacterial pathogenesis and the designing of new drugs against staphylococcal infections. Surprisingly, the exact chemical structure of the CW TA of *S. epidermidis* is still not known. Archibald et al.[8] in 1968 investigated the CW TA of *S. epidermidis* I2. It was shown to be a (1 → 3)-linked poly(glycerol phosphate), containing β-glucopyranosyl and D-alanyl substituents. According to the authors, about a half of glycerol residues were glucosylated at the 2-position. The D-alanyl substitution was tentatively assigned to the glycerol residues that are not glucosylated. Later, the structure of a number of CW TA of staphylococci was elucidated by Endl et al.[9] after extraction by alkaline hydrolysis and depolymerisation with 70% aqueous hydrofluoric acid, followed by gel filtration and analysis of the released glycosides. For *S. epidermidis* type strain ATCC 14990^T, the poly(glycerol phosphate) structure was suggested, with glycerol partially substituted in position 2 with α-Glc and α-GlcNAc. The degree of glucose substitution depended on the glucose content of the growth media. In other *S. epidermidis* strains, β-Glc and β-GlcNAc were also present. *S. epidermidis* strain ATCC 35984 (RP62A) was not included in the study. The possible substitution with alanine, or any other O-esterification—an important feature for the biological function of the CW TA—was not elucidated, since the isolation procedure included an alkaline treatment, sufficient for complete O-deacylation of CW TA.

An interesting feature of the biofilm producing model strain *S. epidermidis* RP62A is its capability to produce some extracellular (EC) TA. Its presence in the extracellular extracts was noticed in an early study of extracellular polymers of this strain.[10] The EC TA may contribute to the highly viscous appearance of the colonies and the strong biofilm formation of this strain.[2] Hussain et al. compared the chemical composition of the extracellular and cell-wall teichoic acids *S. epidermidis* RP62A.[11,12] Both polymers contained glucose, *N*-acetylglucosamine, glycerol, phosphate and D-alanine, but their detailed structure have not been established. In the present study, we describe the detailed chemical structure of the CW and EC TA of the *S. epidermidis* reference biofilm-producing strain RP62A.

2. Results

2.1. Preparation and structural analysis of the CW TA

The CW TA was isolated from the defatted cell walls of *S. epidermidis* RP62A, as described in Section 4, and purified by gel-filtration and ion-exchange chromatography. According to GLC, TLC and colorimetric[13] analysis, it was composed of glycerol, phosphate, D-glucose, D-glucosamine (Glc–GlcN ratio ~5:1) and D-alanine.

The detailed structure of the TA was elucidated by NMR spectroscopy. The ^{1}H and ^{13}C spectra of the TA were fully assigned by 2D homonuclear and heteronuclear correlation techniques. The assignments are summarised in Table 1, and the corresponding structure is shown schematically in Figure 1. Spectra contained signals of one spin system of nonsubstituted α-GlcNAc (unit **B**), two α-Glc (nonsubstituted, unit **A** and acylated with Ala at O-6, unit **F**; see below) and four 1,3 phosphorylated glycerol (Gro) residues: one with free 2-OH, unit **D**; one substituted with Glc or Glc6OAla, unit **C**; one substituted with GlcNAc, unit **E** and a minor variant, unit **G**, possibly acylated with alanine. Linkages between Glc and Gro, and between GlcNAc and Gro were confirmed by NOE (cross peaks from H-1 of the monosaccharide residue to H-2 of Gro) and HMBC (between H-1 of the monosaccharides and C-2 of Gro residues) spectra. The ^{31}P NMR spectrum showed one phosphodiester resonance at 1.54 ppm, which in ^{1}H–^{31}P HMQC spectrum, gave correlations to H-1 and H-3 of all glycerol residues.

The data were in agreement with the previously suggested structure of the CW TA as (1 → 3)-poly(glycerol phosphate), substituted at O-2 of glycerol with single residues of α-Glc and α-GlcNAc. ^{13}C chemical shifts of α-Glc (**A**), α-GlcNAc (**B**) and Gro residues **C**, **E** and **D** (Table 1) are in good agreement with literature data.[14] ^{13}C chemical shifts of the residue **G** closely correspond to the values reported for a (1 → 3)-linked Gro residue substituted at position 2 with an *O*-L-lysyl group.[14]

The most interesting structural feature of the cell-wall TA of *S. epidermidis* RP62A is a partial esterification of the α-Glc residues by D-alanine at C-6. The site of substitution with Ala was determined based on HMBC correlation between Ala C-1 and Glc H-6 (Fig. 2). It agreed with downfield ^{13}C chemical shift of the acylated Glc C-6 and the upfield shift of the C-5 of the α-Glc **F**, compared to its corresponding value in the unsubstituted residue **A** (Table 1 and Fig. 2). These observations gave a clear indication that some of the glucose residues are acylated at C-6. The amount of acylated Glc residues was similar to the amount of GlcNAc in the polymer, judging from H-1 signal intensities. HMBC correlation between H-2 of the residue **G** and alanine carbonyl group was not observed, probably because of its low intensity.

Table 1. ^{1}H and ^{13}C chemical shifts of CW and EC TA of *S. epidermidis* RP62A

Unit		1/1′	2	3/3′	4	5	6	6′
α-Glc **A**	^{1}H	5.21	3.54	3.79	3.42	3.96	3.79	3.91
	^{13}C	98.8	72.5	74.0	70.7	72.9	61.5	
α-GlcNAc **B**	^{1}H	5.12	3.96	3.83	3.49	3.99	3.79	3.91
	^{13}C	97.8	54.6	72.1	71.1	73.0	61.5	
α-Glc6OAla **F**	^{1}H	5.22	3.57	3.80	3.42	4.22	4.44	4.66
	^{13}C	98.3	72.3	73.8	70.7	70.7	66.3	
Gro **C**	^{1}H	4.00/4.06	4.14	4.00/4.06				
	^{13}C	65.7[a]	76.4	66.3[a]				
Gro **E**	^{1}H	4.00/4.06	4.08	4.00/4.06				
	^{13}C	65.7[a]	76.7	66.3[a]				
Gro **D**	^{1}H	3.89/3.96	4.06	3.89/3.96				
	^{13}C	67.3	70.6	67.3				
Gro2Ala **G**	^{1}H	4.11	5.40	4.11				
	^{13}C	64.6	75.2	64.6				
Ala	^{1}H		4.28	1.62				
	^{13}C	171.5	49.8	16.3				

Spectra were recorded at 500 MHz at 25 °C, chemical shifts are given relative to acetone ($\delta_H = 2.225$ ppm, $\delta_C = 31.5$ ppm). NAc:CH_3 signals at 2.09 (^{1}H) and 23.1 (^{13}C) ppm, CO at 175.5 (^{13}C) ppm.
[a]Assignment can be interchanged inside the residue.

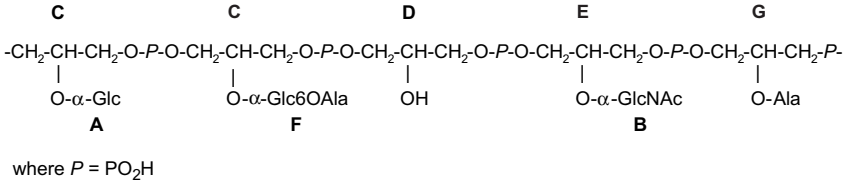

Figure 1. Suggested schematic structure of the CW and EC TA of *S. epidermidis* RP62A.

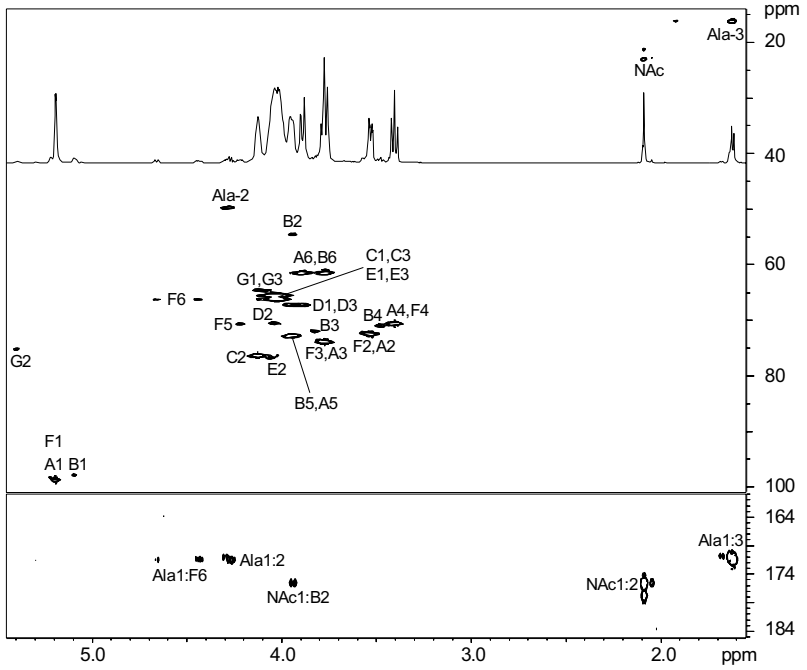

Figure 2. Heteronuclear ^{1}H–^{13}C chemical shift correlation (HSQC) spectrum of the cell-wall TA of *S. epidermidis* RP62A (upper panel) and a fragment of heteronuclear multiple bond correlation (HMBC, lower panel) correlation spectrum showing correlations from carbonyl groups, used for the localisation of alanine.

To confirm the pattern of substitution with alanine, and address the question of whether α-Glc, α-Glc6Ala and α-GlcNAc were present on one polymer chain, or if different polymers carrying only one type of substituents existed, the cell-wall TA was studied by capillary electrophoresis–mass spectrometry (CE–MS). Analysis at high orifice voltage, providing the environment to break a polymer into repeating units, gave diagnostic fragments arising from cleavage of phosphodiester bonds (Table 2). Most abundant were fragments containing Glc, Gro and phosphate, indicating that the GroP(Glc) fragment was predominant in the polymer chain of TA. Fragments containing simultaneously Glc, GlcN, and Glc and Ala, together with Gro and phosphate, were also identified. The tandem mass-spectrometry (MS–MS) spectra of the fragments at m/z 672 and 826 confirmed their assignment as GroP(Glc)-GroP(GlcNAc) and GroP-GroP(Glc)-GroP(GlcNAc), respectively (masses are $M-H_2O$; Fig. 3a). Precursor ion scan of m/z 356 (GroPGlcN) shows fragments containing glucose [m/z 672; GroP(Glc)-GroP(GlcNAc)] confirming that Glc and GlcNAc are present in the same polymer chain (Fig. 3b). Similarly, precursor ion scan of m/z 386 (GroPGlcAla) shows fragments at m/z 702 ($Glc_2Gro_2P_2Ala$), and 939 ($Glc_3Gro_3P_2Ala$), unambiguously indicating that glycerol residues substituted with Glc and Glc6Ala are present in the same polymer chain (Fig. 3c).

Table 2. CE–MS analysis of CW TA of *S. epidermidis* RP62A at high orifice voltage. Observed secondary ions (m/z) and their proposed composition[a]

Observed ions (m/z)	Proposed composition
315[b]	GroP(Glc)
356	GroP(GlcNAc)
386[b]	GroP(GlcAla)
469[c]	GroP(Glc)-GroP
510[d]	GroP(Glc*N*Ac)-GroP
551[b]	GroP(Glc)-Gro(Glc)
631[c]	GroP(Glc)-GroP(Glc)
672[b,d]	GroP(Glc)-GroP(GlcNAc)
702[e]	GroP(Glc)-GroP(GlcAla)
825[b]	GroP-GroP(Glc)-GroP(GlcNAc)
867[b,c]	GroP(Glc)-GroP(Glc)-Gro(Glc)
939[b,c,e]	GroP(Glc)-GroP(GlcAla)-GroP(Glc)
1264[b]	GroP(Glc)-GroP(Glc)-GroP(Glc)-GroP(Glc)

[a] Average mass units were used for calculation of molecular weight based on proposed composition as follows: Gro, 73; *P*, 79.98; Glc, 162.15; Glc*N*Ac, 203.19; Ala, 71.07.

[b] Composition confirmed by tandem mass spectrometry (MS–MS) analyses.

[c] Confirmed by precursor ion spectrum of m/z 315 corresponding to a fragment GroPGlc.

[d] Confirmed by precursor ion spectrum of m/z 356 corresponding to a fragment GroPGlcNAc.

[e] Confirmed by precursor ion spectrum of m/z 386 corresponding to a fragment GroPGlcAla.

2.2. Preparation and structural analysis of the EC TA

In order to maximise the yield of the EC TA, *S. epidermidis* RP62A was grown in BHI medium. Crude cell-associated matrix was extracted by sonication in 0.9% NaCl on ice, essentially as described in Ref. 15, in conditions excluding cells lysis. The integrity of bacterial cells was verified by viable counts before and after sonication. Therefore, contamination of extracellular with the cell-wall TA was minimised. No DNA was present in the crude saline extract, thus confirming that cell lysis did not take place (data not shown).

From the crude extract, proteins were removed by TCA precipitation, followed by proteinase treatment and extraction with water-saturated phenol and phenol–$CHCl_3$ mixture. This mild deproteinisation method, widely used for the purification of nucleic acids, was proved here to be efficient for the purification of the EC TA of *S. epidermidis* RP62A. Great care was taken to avoid treatment with any buffers with pH > 7.0, in order to minimise losses of ester-linked substituents. Presence of the alanine substitution was checked by TLC after O-deacylation of EC TA. The purified EC TA was obtained following gel-filtration and ion-exchange chromatography, as described in Section 4, and analysed by NMR spectroscopy. The same structure as described above for CW TA was identified (Table 1). However, the distribution of alanine residues was quantitatively different, most of them being at O-2 of glycerol **G** and less at O-6 of Glc residues **F**.

3. Discussion

1,3-Poly(glycerol phosphates) are one of the most widely occurring bacterial CW TA. They may be substituted at O-2 of glycerol with glucose, galactose, N-acetylated aminosugars; as well as D-alanyl, L-lysyl or acetyl residues.[16]

In an earlier study, it was shown that alanine substitution in wall TA of *S. aureus* decreases the binding of magnesium ions, which form bridges across phosphate groups in TA chains lying adjacent to each other.[17] It was concluded that alanine amino groups compete with Mg^{2+} for the anionic centres along the polyol phosphate chain. Baddiley[18] recently discussed the role of teichoic acids in bacterial aggregation. He suggested that in case of the TA lacking alanine residues, both valencies of each Mg^{2+} (Ca^{2+}) would be firmly associated with phosphate groups within the polymer chain and there would be no interaction with external anionic centres. When positively charged alanine ester residues are present, the less firmly associated Mg^{2+} (Ca^{2+}) would be able to attract other anionic centres, such as an anionic centre in the adhesin. Thus, in bacteria undergoing

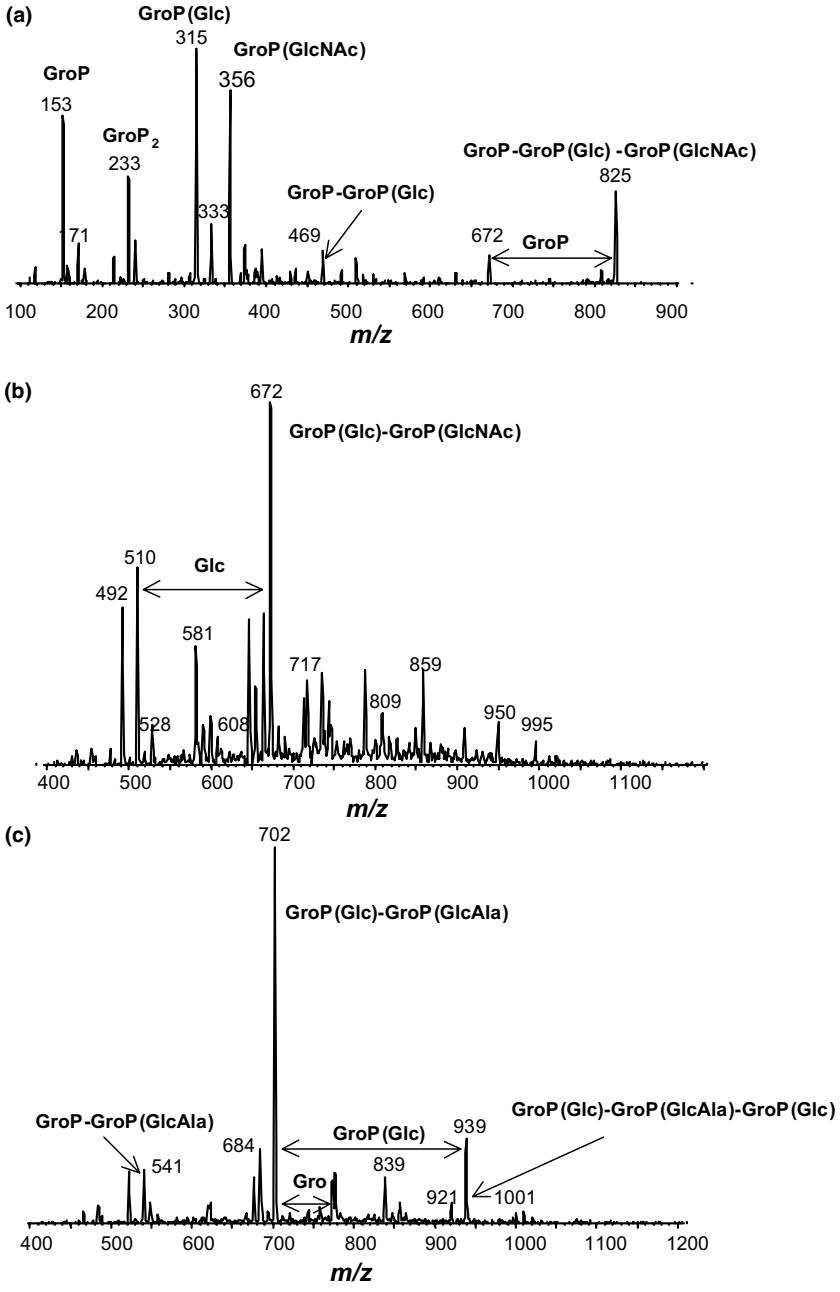

Figure 3. Negative-ion capillary electrophoresis–mass spectrum (CE–MS) of CW TA from *S. epidermidis* RP62A. (a) MS/MS of m/z 825 corresponding to GroP-GroP(Glc)-GroP(GlcNAc) fragment; (b) precursor ion spectrum of m/z 356, corresponding to GroP(Glc) fragment; (c) precursor ion spectrum of m/z 386, corresponding to GroP(GlcAla) fragment.

coaggregation or colonising an artificial surface, a divalent cation could be shared between a phosphate in the TA chain and an anionic centre of an adhesin or, perhaps, on a solid surface directly. It is tempting to speculate that the positive charge of alanine esters, present at C-6 of α-Glc residues side chain, are relatively distanced from the poly(glycerol phosphate) backbone of the TA of the biofilm producing strain *S. epidermidis* RP62A, and can therefore participate more easily in the process of bacterial adherence.

The structure of the cell-wall teichoic acids of staphylococci was largely elucidated in the 60–70th years (see, for example, Refs. 8,19). The corresponding structural data entered in textbooks of biochemistry and microbiology. It is important to point out, however, that the site of substitution with alanine in *S. epidermidis* was assigned only tentatively. To date, the study of Endl et al.,[9] where O-acyl substitution is not discussed, still remains the reference for structures of cell-wall teichoic acids of the genus *Staphylococcus*.

D-Alanyl substitution at O-6 of glucose along with O-2 of glycerol, found in this study for the CW and EC TA of *S. epidermidis* RP62A seems to be a novel structural feature. With staphylococci emerging now as a major cause of nocosomial infection, and taking into account the important role TA are playing in the biofilm formation, closely related to the pathogenesis of these bacteria, our results suggest that structures of teichoic acids of other Staphylococci could be re-investigated and refined on the basis of modern methods of structural analysis.

4. Experimental

4.1. Bacterial strain and culture conditions

S. epidermidis RP62A (ATCC 35984) is a generous gift of Prof. Gerald Pier (Harvard Medical School, Boston, MA, USA).

The cells were grown at 37 °C for 24 h in 4 L of Tryptic Soy Broth (TSB, Difco) in four 3-L Erlenmeyer flasks, each inoculated with 20 mL of a 20 h culture in the same medium and shaking at 85 rpm. For the preparation of the EC TA, cells were grown in 12 L of Brain Heart Infusion broth (BHI, Difco) in four 3-L Erlenmeyer flasks with moderate shaking (60 rpm).

4.2. Preparation of CW and EC TA

Bacterial cells were collected by centrifugation (5000*g*, 4 °C, 20 min), suspended in 0.9% NaCl (pH 6), sonicated on ice (IKASONIC sonicator, 230 V, 50/60 Hz IKA Labothechnik, Germany at 50% amplitude, cycle 0.5; 4×30 s) to remove the cell-associated extracellular material, and collected by centrifugation. Supernatant was clarified by short high-speed centrifugation (12,000*g* for 15 min at 4 °C), filter-sterilised and used for the extraction of EC TA. The extraction of CW TA was carried out essentially as described in Ref. 20 for the selective extraction of CW TA from *Enterococcus faecalis*. Briefly, cells were suspended in 4% sodium dodecyl sulfate (SDS) and boiled for 30 min to eliminate the lipoteichoic acid and other lipophylic material. Cells were collected by centrifugation at room temperature, and the pellet was washed six times with distilled water to remove SDS. Cells were then broken with glass beads (0.5 g/g wet cells) in Edmund Bühler homogeniser at 4 °C for 5 min. Glass beads were removed by sedimentation, and the cell walls collected by centrifugation (12,000*g*, 20 min). Pellets were then washed twice with distilled water, and stirred with 5% TCA at 4 °C for 24 h. Insoluble material was removed by centrifugation (12,000*g*, 20 min), supernatant dialysed extensively against distilled water and freeze-dried. The resulting product was re-dissolved in 5% TCA, any insoluble material was removed by centrifugation. Clear supernatant was purified on a Sephadex G-50 column. Average yield of CW TA was 1–1.3 mg per 1 g of wet bacterial cell. For detailed NMR analysis, 5 mg of CW TA was purified by ion-exchange chromatography on Hitrap Q column (QAE-Sepharose, Amersham).

To the crude saline extract, TCA was added to a concentration of 5%. The precipitate was removed by centrifugation, the clear supernatant concentrated on an Amicon® Ultrafiltration Cell with a 10 kDa molecular cutoff membrane (Millipore), dialysed and lyophilised. The residue was re-dissolved in 4 mL of water and treated with Proteinase K (Sigma, 0.1 mg/mL) at 50 °C for 3 h. The mixture was extracted with water-saturated phenol (3×) and 1:1 phenol–$CHCl_3$ (3×). The water phase was applied to a Sephadex G-50 column, irrigated with water. EC TA, eluted in the void volume of the column, was collected and lyophilised. It was further purified on an ion-exchange column as described above for the CW TA.

4.3. General and analytical methods

Glycerol and monosaccharides were identified by GLC on a Shimadzu GC-14 B gas chromatograph equipped with a flame ionisation detector and a Zebron ZB-5 capillary column (30 m×0.25 mm), with hydrogen as carrier gas, using a temperature gradient 170 °C (3 min), 260 °C at 5 °C/min.

Prior to analysis, samples of TA were depolymerised by 48% hydrofluoric acid (HF, Acros Organic) for 48 h at 4 °C. HF was evaporated under a stream of nitrogen, the residue hydrolysed with 4 M TFA (120 °C, 2 h) and converted into alditol acetates by conventional methods.

The presence of alanine in EC TA preparations was confirmed by thin layer chromatography (TLC), following the O-deacylation of the samples with 10% NH_4OH at room temperature overnight. The absolute configuration of alanine in the EC TA was determined by comparison of the GLC (program b) retention time of its *N*-acetyl-(*R*)-2-butyl ester with authentic standards. The absolute configuration of alanine in CW TA was determined by chiral HPLC of the TA-hydrolysate on Chirex D penicillamine column (250×4.6 mm, Phenomenex) in 2 mM $CuSO_4$ in 15% MeOH with UV detection at 254 nm. The absolute configuration of glucose and glucosamine were determined by the procedure of Gerwig et al.[21]

Gel permeation chromatography was carried out on a Sephadex G-50 column (1.6×95 cm; Pharmacia), irrigated with water. Fractions (5 mL) were assayed colorimetrically for aldose[22] and aminosugars.[23]

Ion-exchange chromatography was carried out on Hitrap Q anion-exchange column containing 5 mL of Q-Sepharose Fast Flow (Amercham Pharmacia Biotech) in a gradient of water—1 M NaCl over 1 h, fractions

containing TA were identified by charring of the eluate spots on TLC plate with 5% H_2SO_4. The product was desalted by gel chromatography on Sephadex G-50 column.

TLC was carried out on Silica Gel 60 plates using 4:1:1 BuOH–HOAc–water. Spots were visualised by spraying with ninhydrin solution (1% in acetone) and heating at 110 °C.

Mass spectrometry. All MS experiments were performed as previously described in details.[24] Briefly, a crystal Model 310 CE instrument (ATI Unicam, Boston, MA, USA) was coupled to an API 3000 mass spectrometer (Perkin–Elmer/Sciex, Concord, Canada) via a microionspray interface. A sheath soln (2:1 isopropanol–MeOH) was delivered at a flow rate of 1 μL/min. An electrospray stainless steel needle (27 gauge) was butted against the low dead vol tee and enabled the delivery of the sheath soln to the end of the capillary column. The separation was obtained on a bare fused-silica capillary on about 90 cm length using 30 mM morpholine/formate in deionised water, pH 9.0, containing 5% MeOH. A voltage of 25 kV was typically applied at the injection. The outlet of the capillary was tapered to ca. 15 μm i.d. using a laser puller (Sutter Instruments, Novato, CA, USA). Mass spectra were acquired with an orifice voltage of −200 V, and dwell times of 3 ms per step of 1 m/z unit in full mass-scan mode. Fragment ions formed by collision activation of selected precursor ions with nitrogen in RF-only quadrupole collision cell, were mass-analysed by scanning the third quadrupole. Collision energies were typically 20–60 eV (laboratory frame of reference).

NMR spectroscopy. NMR spectra were recorded at 25 °C in D_2O on a Varian UNITY INOVA 500 instrument, using acetone as reference (^1H, δ 2.225 ppm; ^{13}C, δ 31.5 ppm). Varian standard programs COSY, NOESY (mixing time of 200 ms), TOCSY (spinlock time 120 ms), HSQC and gHMBC (evolution delay 100 ms) were used. ^1H–^{31}P HMQC correlation spectra were recorded on Varian UNITY INOVA 400 instrument.

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