

# The structure of the extracellular teichoic acids from the allergy-protective bacterium *Lactococcus lactis* G121\*

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## Abstract

The Gram-positive bacterium *Lactococcus lactis* G121 is a farm isolate that protects mice from ovalbumin-induced asthma. To understand the molecular mechanisms of such allergy-protective properties, the isolation and characterization of cell envelope constituents is crucial. Here, structural analyses of the extracellular teichoic acid (EC TA) from *L. lactis* G121 are presented. Extraction with 0.9% saline afforded a crude TA fraction. Consecutive size exclusion chromatography on Biogel P60 and P10 matrix was performed to purify the sample. Chemical component analyses, high-resolution electrospray ionization Fourier-transformed ion cyclotron mass spectrometry, and nuclear magnetic resonance spectroscopy were conducted for structural elucidation. The EC TA was a poly(glycosylglycerol phosphate) molecule with a repeating unit of  $-6)-[\beta\text{-D-Glcp}-(1\rightarrow3)-][\alpha\text{-D-GlcpNAc}-(1\rightarrow4)-]\alpha\text{-D-GalpNAc}-(1\rightarrow3)-\beta\text{-D-GlcpNAc}-(1\rightarrow2)\text{-glycerol}-(1\text{-P-})$ .

**Keywords:** asthma prevention; ESI FT-MS spectrometry; extracellular teichoic acid; *Lactococcus lactis*; NMR spectroscopy.

## Introduction

During the last two decades, several studies in Europe, Australasia, and North America unambiguously demonstrated

the anti-allergic features of the farming lifestyle, in particular that children growing up on traditional farms are protected from allergic sensitization, hay fever, and asthma (both the atopic and non-atopic types) (von Mutius and Vercelli, 2010). The main important farm stimuli involved are contact with animals (mainly cattle), fodder (e.g., hay, silage), and raw milk consumption. Further detailed investigations of farm environments identified a cow-shed dust extract (Peters et al., 2006; Gorelik et al., 2008) and from this, grass arabinogalactan as allergy-protective components (Peters et al., 2010). Additionally, two bacterial species, namely Gram-negative *Acinetobacter lwoffii* F78 and Gram-positive *Lactococcus lactis* G121, were isolated from cow-sheds and shown to possess allergy-protective properties in an ovalbumin-provoked mouse asthma model (Debarry et al., 2007; Hanuszkiewicz et al., 2008a,b; Fischer et al., 2011).

Priming of children occurs between the prenatal phase and the early years of life. In the PARSIFAL study, it was proven that maternal farming during pregnancy upregulated the cluster of differentiation (CD) 14, Toll-like receptor (TLR) 2, and TLR4 mRNA levels in school-age children. Interestingly, this effect was correlated with the number of animal species the mother had faced during pregnancy (Ege et al., 2006). Furthermore, consumption of raw milk products by the mother yielded an increase in pro-inflammatory cytokines as interferon (IFN)- $\gamma$  or tumor necrosis factor (TNF)- $\alpha$  in the cord blood of farm children (Pfefferle et al., 2010). Beyond that, latest research has proven that genetic properties and their alteration by environmental factors also contribute to the allergic outcome of an individual. In a recent study, Brand et al. (2011) demonstrated the IFN- $\gamma$ -dependent abolishment of bronchial asthma in murine progeny by maternal exposure to *A. lwoffii* F78. Contact to this microbe, in which LPS appears to be the main molecule responsible for its allergy-protective properties (Debarry et al., 2010), maintained acetylation of histone 4 at the *IFNG* promoter in CD4<sup>+</sup> cells of the offspring, which correlated with the IFN- $\gamma$  expression (Brand et al., 2011).

The initial model of a T<sub>H</sub>1-skewed innate immune response due to early contact with environmental agents is currently only one out of various others regarding, e.g., adaptive immunity (Schaub et al., 2008, 2009), epigenetics (Renz et al., 2011), the human microbiome, or certain parasitoses (Danilowicz-Luebert et al., 2011). To understand the molecular basis of the allergy protection mediated by *L. lactis* G121, including host immune receptor(s) and signaling route(s), its cell envelope compounds need to be structurally and functionally characterized. Recently, the lipoteichoic acids (LTAs) of *L. lactis* G121 were investigated and found to be built up of a poly(*sn*-glycerol 1-phosphate) main chain

\*Dedicated to Prof. Dr. Rudolf Geyer on the occasion of his 65th birthday.

non-stoichiometrically substituted at the *O*-2 of glycerol by either D-Ala or  $\alpha$ -D-Galp. Its lipid anchor was  $\alpha$ -D-Glcp-(1 $\rightarrow$ 2)- $\alpha$ -D-Glcp-(1 $\rightarrow$ 3)-1,2-diacyl-*sn*-glycerol. An LTA-mediated IL-6 enhancement in mononuclear cells was shown to be neither TLR2 nor TLR4 dependent (Fischer et al., 2011). In this article, the structure of the EC TA of *L. lactis* G121 is presented.

## Results

### Isolation and composition of ECTA

EC TA was isolated by extraction of dry bacteria with 0.9% aqueous NaCl, followed by size-exclusion chromatography (SEC), in a yield of 4.6% of the dry biomass.

Compositional analyses of the isolated sample identified L-Rha (525.8 nmol/mg), D-GalN (not quantified), D-GlcN (not quantified), D-Glc (329.4 nmol/mg), phosphate (P, 1  $\mu$ mol/mg), and Gro (not quantified). Fatty acid analysis proved that EC TA was free of lipophilic substances.

### Mass spectrometry

The composition of the EC TA repeating units was assigned by high-resolution electrospray ionization Fourier-transformed ion cyclotron mass spectrometry (ESI FT-ICR-MS). Figure 1 depicts the charge-deconvoluted spectrum obtained in the negative ion mode under soft-ionization conditions in which eight different species could be distinguished with regard to the number of repeating units. One repeat comprised one P,

one hexose (Hex), three *N*-acetyl hexosamines (HexNAc), and one Gro with a mass of 925.2941 u ( $H_2O$  subtracted). Table 1 summarizes all assigned EC TA masses. The multiplicity of certain repeat signals could be ascribed to  $Na^+$  and/or  $K^+$  adducts and fragments of the EC TA.

### NMR spectroscopy

The 1D  $^1H$  NMR spectrum of EC TA (Figure 2) revealed three major anomeric signals. Full assignment of 2D homonuclear and heteronuclear correlation experiments identified a fourth spin system, the H-1 of it being saturated with the HOD signal. The anomeric proton signals were defined as H-1 of  $\alpha$ -D-GalpNAc [residue A,  $\delta_H$  5.60,  $^1J_{1,2}$  3.85 Hz, H-1/H-2 coupling in the ROESY spectrum (Figure 3) at  $\delta_H$  5.60/4.49], H-1 of

**Table 1** Assignments of *L. lactis* G121 EC TA mass peaks from ESI-MS analyses.

| Detected mass (u) | Composition                                     |
|-------------------|-------------------------------------------------|
| 1868.606          | 2 P, 2 Hex, 6 HexNAc, 2 Gro=2 repeats           |
| 2793.883          | 3 P, 3 Hex, 9 HexNAc, 3 Gro=3 repeats           |
| 3741.172          | 4 P, 4 Hex, 12 HexNAc, 4 Gro+ $Na^+$ =4 repeats |
| 4644.473          | 5 P, 5 Hex, 15 HexNAc, 5 Gro=5 repeats          |
| 5569.811          | 6 P, 6 Hex, 18 HexNAc, 6 Gro=6 repeats          |
| 6495.081          | 7 P, 7 Hex, 21 HexNAc, 7 Gro=7 repeats          |
| 7420.370          | 8 P, 8 Hex, 24 HexNAc, 8 Gro=8 repeats          |
| 7442.429          | 8 P, 8 Hex, 24 HexNAc, 8 Gro+ $Na^+$ =8 repeats |
| 8367.629          | 9 P, 9 Hex, 27 HexNAc, 9 Gro+ $Na^+$ =9 repeats |

Average mass units of 74.04 for Gro, 79.97 for P, 162.05 for Hex, and 203.04 for HexNAc were used for calculations.

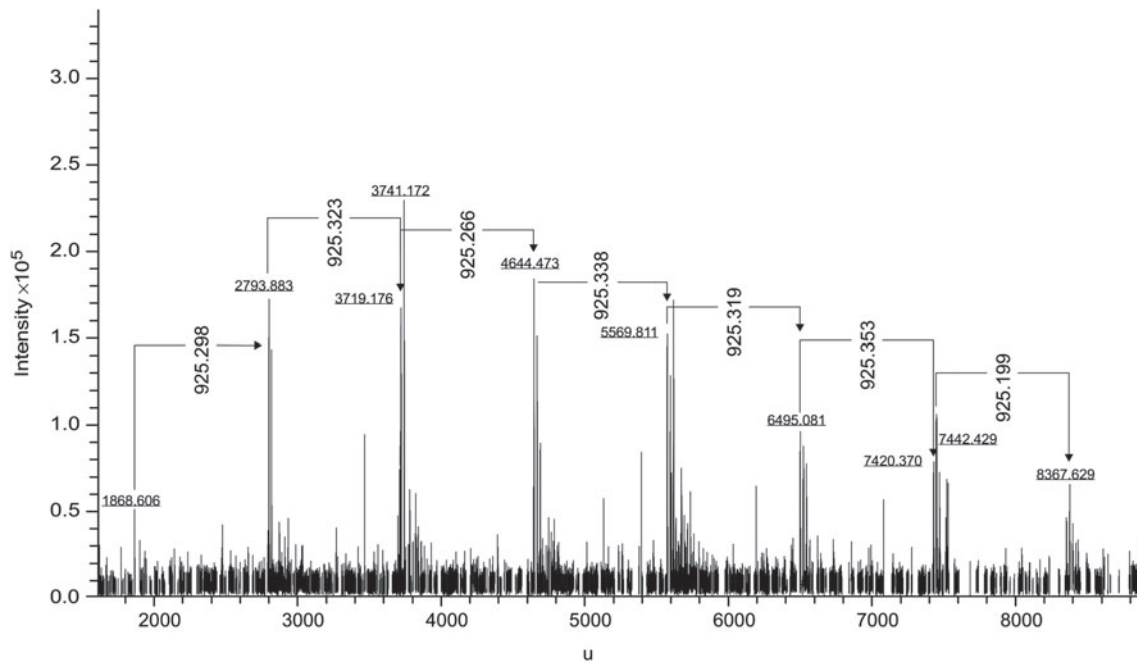


**Figure 1** ESI FT-MS spectrum of the EC TA isolated from *L. lactis* G121. The monoisotopic peaks from two (1868.6061 u) to nine repeating units (8367.6294 u) are presented. One repeat was found to consist of one P, one Hex, three HexNAc, and one Gro with a mass of 925.2941 u ( $H_2O$  subtracted). Unlabeled peaks refer to  $Na^+$  and/or  $K^+$  adducts of the EC TA as well as fragments of it. A summary of the detected mass peaks is given in Table 1.

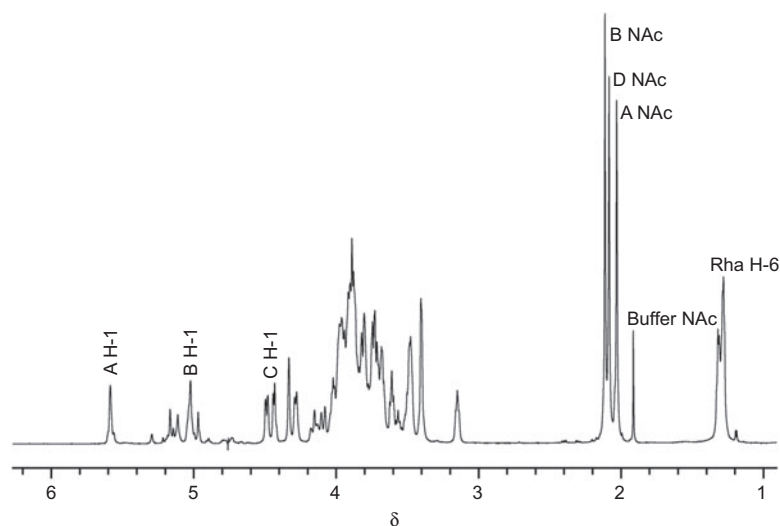


**Figure 2** 1D  $^1\text{H}$  NMR spectrum of EC TA polymer of *L. lactis* G121.

Measurements were carried out in  $\text{D}_2\text{O}$  at 700 MHz and 27°C. The major anomeric protons are indicated by capital letters as explained in Table 2. Chemical shifts were calibrated to external acetone ( $\delta_{\text{H}}$  2.225;  $\delta_{\text{C}}$  31.45).

$\alpha$ -D-GlcpNAc (residue B,  $\delta_{\text{H}}$  5.03, cross peak of H-1/H-2 in the ROESY spectrum at  $\delta_{\text{H}}$  5.03/3.93), H-1 of  $\beta$ -D-Glcp (residue C,  $\delta_{\text{H}}$  4.44,  $^1J_{1,2}$  7.96 Hz), and H-1 of  $\beta$ -D-GlcpNAc (residue D,  $\delta_{\text{H}}$  4.74,  $^1J_{1,2}$  8.22 Hz). The HMBC experiment identified correlations between proton/carbon resonances A3/C1, A4/B1, and A1/D3, indicating the glycosidic bonds between these sugars. The connections were further affirmed by a ROESY experiment shown in Figure 3, which revealed inter-residual connectivities between C-1/A-3 ( $\delta_{\text{H}}$  4.44/3.87),

B-1/A-4 ( $\delta_{\text{H}}$  5.03/4.34), and A-1/D-3 ( $\delta_{\text{H}}$  5.60/3.81). The linkage to Gro (residue E) could be identified by the NOE connectivity between proton signals D-1 and E-2 ( $\delta_{\text{H}}$  4.74/3.97). Strong downfield shifts of carbons A3, A4, D3, and E-2 (Table 2) confirmed all substitutions. In a  $^1\text{H}$ ,  $^{31}\text{P}$  HMQC experiment, crossings were detected between a phosphodiester signal at  $\delta_{\text{P}}$  0.76 with those of protons E-1 ( $\delta_{\text{H}}$  3.99) as well as A5 ( $\delta_{\text{H}}$  4.03) and A6 ( $\delta_{\text{H}}$  3.96). Three *N*-acetyl- $\text{CH}_3$  resonances could be assigned to the corresponding sugars by the HMBC

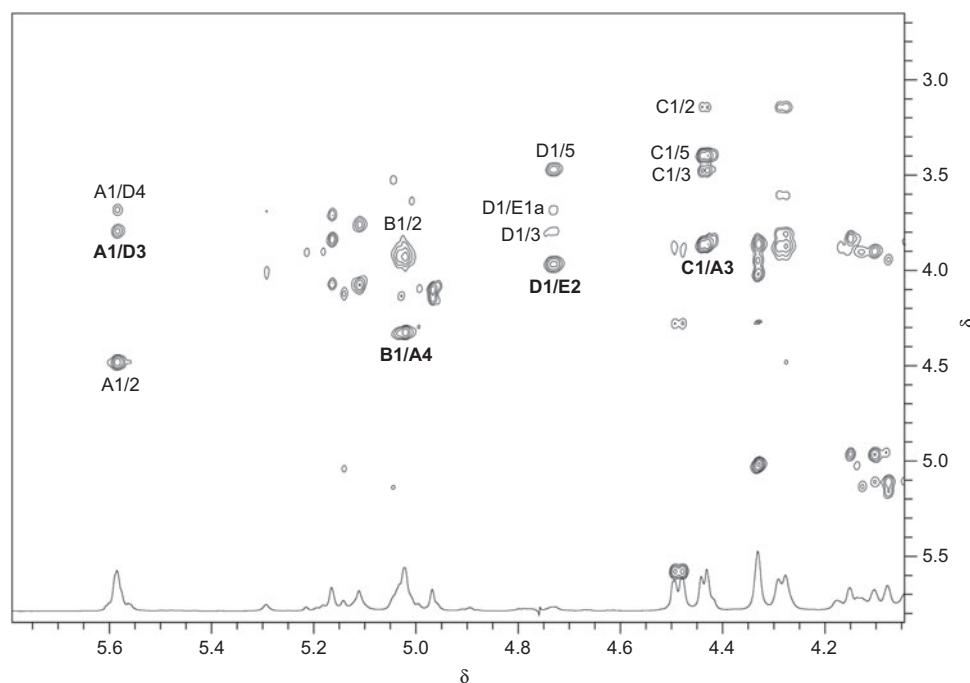


**Figure 3** Overlay of  $^1\text{H}$  and  $^1\text{H}$ ,  $^1\text{H}$  ROESY NMR spectra of the EC TA polymer recorded at 700 MHz in  $\text{D}_2\text{O}$  at 27°C.

Signals were calibrated to external acetone ( $\delta_{\text{H}}$  2.225;  $\delta_{\text{C}}$  31.45). Capital letters are defined in Table 2 and refer to the residues depicted in Figure 4. Arabic numerals refer to the respective proton. Unlabeled minor cross peaks could be linked to contaminating L-rhamnan signals. Bold letters/numerals indicate the glycosidic bonds between the residues.

**Table 2** Chemical shifts from NMR analyses of the native EC TA polymer of *L. lactis* G121 at 600 MHz.

| Residue              | Chemical shifts of protons and carbons ( $\delta$ ) |               |                  |               |               |                       |               |
|----------------------|-----------------------------------------------------|---------------|------------------|---------------|---------------|-----------------------|---------------|
|                      | 1 a/b                                               | 2             | 3 a/b            | 4             | 5             | 6 a/b                 | NAc           |
| $\alpha$ -D-GalNAc A | H 5.60 (5.48)                                       | 4.49 (4.48)   | 3.87 (3.91)      | 4.34 (4.29)   | 4.03 (3.94)   | 3.96 (3.67)           | 2.03 (2.02)   |
|                      | C 97.90 (98.38)                                     | 49.80 (49.96) | 77.20 (77.09)    | 75.70 (75.60) | 72.0 (72.93)  | 64.60 (60.68)         | 23.10 (23.05) |
| $\alpha$ -D-GlcNAc B | H 5.03 (4.99)                                       | 3.93 (3.89)   | 3.88 (3.89)      | 3.61 (3.60)   | 4.28 (4.29)   | 3.83/3.88 (3.80/3.87) | 2.08 (2.07)   |
|                      | C 98.30 (98.21)                                     | 55.10 (55.08) | 71.60 (71.44)    | 70.60 (70.54) | 72.90 (72.62) | 61.10 (61.01)         | 22.80 (23.15) |
| $\beta$ -D-Glc C     | H 4.44 (4.44)                                       | 3.16 (3.14)   | 3.48 (3.45)      | 3.41 (3.38)   | 3.41 (3.38)   | 3.74/3.90 (3.73/3.90) |               |
|                      | C 106.0 (105.97)                                    | 74.20 (74.08) | 76.80 (76.76)    | 70.90 (70.88) | 77.0 (76.95)  | 61.80 (61.71)         |               |
| $\beta$ -D-GlcNAc D  | H 4.74 (4.71)                                       | 3.81 (3.80)   | 3.81 (3.75)      | 3.69 (3.70)   | 3.48 (3.45)   | 3.74/3.90 (3.73/3.90) | 2.06 (2.05)   |
|                      | C 102.10 (101.89)                                   | 55.50 (55.50) | 78.20 (78.96)    | 72.50 (72.13) | 76.80 (76.76) | 61.80 (61.71)         | 23.50 (23.51) |
| Gro E                | H 3.68/3.73 (3.62/3.65)                             | 3.97 (3.85)   | 3.99 (3.66/3.71) |               |               |                       |               |
|                      | C 61.90 (62.03)                                     | 81.0 (82.37)  | 65.80 (62.37)    |               |               |                       |               |

Signals of the EC TA monomer after depolymerization with 48% HF were measured at 700 MHz and are displayed in brackets. Signals are sketched in the  $^1\text{H}/^1\text{H}, ^{13}\text{C}$  HSQC-DEPT overlay Figure 5, and the structure of the repeating unit is reported in Figure 4.

experiment by correlations between the ring H-2 and carbonyl carbons. Taking chemical, ESI-MS, and NMR data together, the structure of the EC TA repeat was as depicted in Figure 4.

In addition, L-Rha signals of lower intensity were identified in the proton spectrum, in particular its characteristic H-6 high field shift of the 6-deoxy group at  $\delta_{\text{H}}$  1.29 and  $\delta_{\text{H}}$  1.33. However, no connectivities between Rha and EC TA could be found in the HMBC or NOE experiment, indicating a contamination that was in agreement with the ESI MS data. To ascertain this contamination, the EC TA was depolymerized with 48% HF and separated by SEC, which yielded two fractions. The  $^1\text{H}$  spectrum of the first fraction (390  $\mu\text{g}$ , 12.2% of EC TA) contained all EC TA but still Rha signals, whereas that of the second fraction (210  $\mu\text{g}$ , 6.6% of EC TA) contained only EC TA signals. Detailed studies of the 1D and 2D NMR spectra confirmed the Rha not being covalently linked to EC TA. Figure 5 displays the  $^1\text{H}, ^{13}\text{C}$  HSQC-DEPT spectrum of the EC TA monomer obtained after HF depolymerization together with its 1D  $^1\text{H}$  spectrum. All chemical shifts are summarized in Table 2.

## Discussion

Since the discovery and structural characterization of the first wall teichoic acid (WTA) in the 1960s, quite a number

of different molecules have been described. In most cases, 1,5-poly(D-ribitol phosphate)- and 1,3-poly(glycerol phosphate)-type WTAs were identified. Substitutions of these polyols show a huge variety; however, D-alanylation and glycosylation by, e.g.,  $\alpha$ - or  $\beta$ -D-GlcNAc (in *Staphylococcus aureus*) or  $\alpha$ -D-Glcp (in *Bacillus subtilis*) are common. Quite often, one bacterial species possesses different types of TA. More exceptional are arabinol-phosphate (*Agromyces cerinus*) or erythritol-phosphate (*Glycomyces tenuis*) units (Neuhaus and Baddiley, 2003; Swoboda et al., 2010). Unlike the LTAs, WTAs are covalently bound to the peptidoglycan (PGN) through a phosphodiester linkage to O-6 of N-acetyl muramic acid. The rather loosely associated EC TA represents WTA that is shed from the PGN, and therefore, structures are usually the same. This has been shown, for example, in *Staphylococcus epidermidis* RP62A and *S. aureus* MN8m (Sadovskaya et al., 2004; Vinogradov et al., 2006). Preliminary data (not presented) indicated that the WTA structure of *L. lactis* G121 is very similar to that of the EC TA.

For *L. lactis*, no TA structures have been reported thus far. Applying high-resolution ESI FT-MS and NMR experiments, an EC TA was identified in strain G121, comprising repeating units of poly(glycosylglycerol phosphate), as shown in Figure 4. Up to nine repeats could be observed by MS analysis (Figure 1).

With regard to the biological function of TAs, it was shown for the model organism *S. aureus* that they neither activated monocytic cell lines to produce cytokines nor stimulated HEK293 cells transfected with TLR2; thus, they do not represent a pathogen-associated molecular pattern in the common sense (Weidenmaier et al., 2010). We further extended these analyses and could also see that the EC TA from *L. lactis* G121 lacks the capacity to stimulate cells through TLR2, TLR4, and the intracellular pattern-recognition receptors NOD1 and NOD2 (data not shown), indicating a very high purity of our preparation. However, WTAs strongly influence bacterial adherence to host tissues or artificial surfaces such as catheters. In nosocomial *S. aureus*

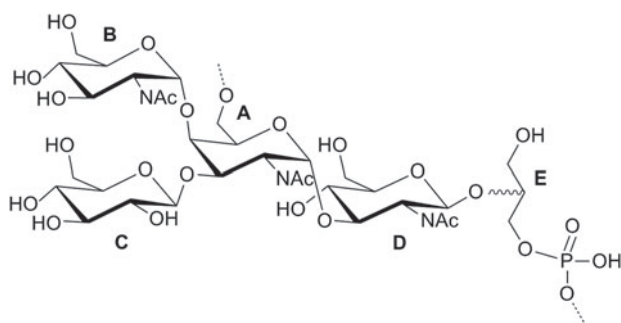


**Figure 4** Structure of the EC TA extracted from *L. lactis* G121. Capital letters are explained in Table 2.

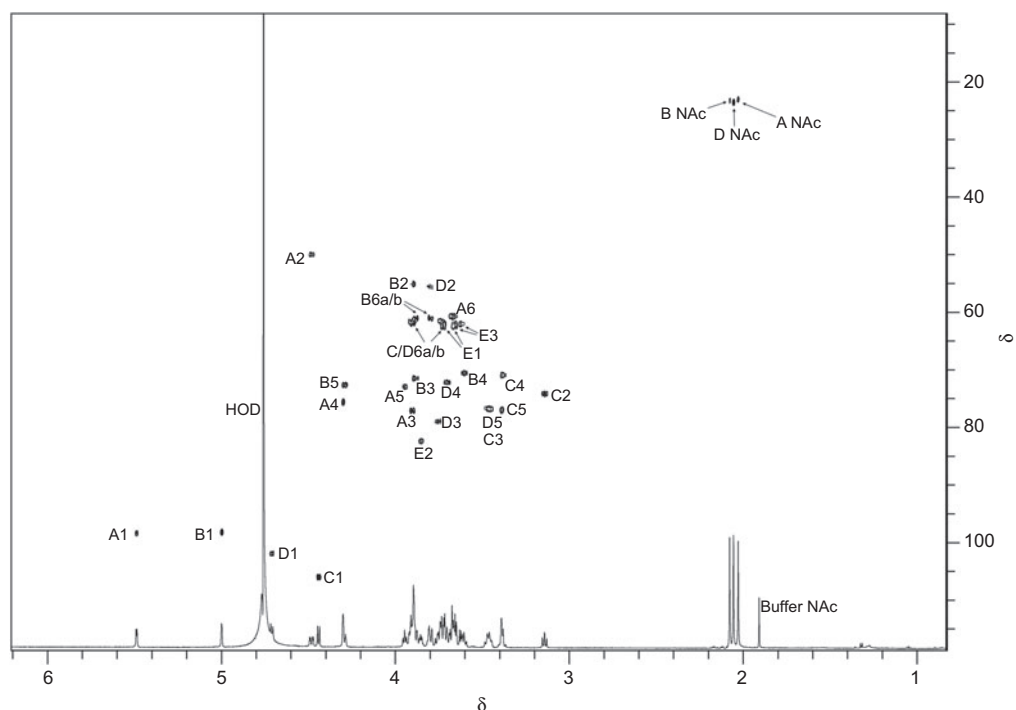


**Figure 5** Overlay of  $^1\text{H}$  and  $^1\text{H},^{13}\text{C}$  HSQC-DEPT NMR spectra of the EC TA monomer after 48% HF hydrolysis. Capital letters are as in Table 2 and refer to the sugars illustrated in Figure 4, and Arabic numerals refer to the respective proton/carbon atom. Spectra were recorded at 700 MHz in  $\text{D}_2\text{O}$  at  $27^\circ\text{C}$  and signals were calibrated to external acetone ( $\delta_{\text{H}}$  2.225;  $\delta_{\text{C}}$  31.45).

or *Enterococcus faecum* infections (Bychowska et al., 2011), WTAs were considered the major virulence factors because they enabled biofilm formation, thus colonization of the gut or nasal epithelium as well as the endothelium. This was supported by data showing that WTA-lacking *S. aureus* were defective in the colonization of cotton rats (Weidenmaier et al., 2004). Further studies are required to characterize if WTA from *L. lactis* G121 does play a similar role.

## Materials and methods

### Bacterial growth

*L. lactis* G121 was cultivated on trypticase soy broth (TSB) agar dishes (supplemented with 0.3% yeast extract; BD, Heidelberg, Germany) and in TSB fluid cultures at  $37^\circ\text{C}$  and agitation at 120 rpm. Precultures were diluted (1:50, by vol) into the main batch.

### ECTA extraction and purification

Incubation of intact cells (5.3 g dry biomass from 10 l) with sterile 0.9% aqueous NaCl at 100 rpm and  $22^\circ\text{C}$  for 6 h was accomplished. The supernatant (10 000 g,  $4^\circ\text{C}$ , 45 min) was dialyzed (16 ku cut-off) against 0.02% sodium azide in water for 3 days, lyophilized, and separated by SEC first on a column (80 $\times$ 2.5 cm) of Biogel P60 and then on a column (80 $\times$ 2.5 cm) of Biogel P10 with pyridinium acetate (50 mM, pH 4.7) as eluent (24.7 mg, 4.6% of the dry biomass). A refractive index detector (Knauer, Berlin, Germany) was used for detection of the fractions.

### ECTA depolymerization

For investigation of EC TA monomers, 3.2 mg of sample was hydrolyzed with aqueous 48% HF (100  $\mu\text{l}$ ,  $4^\circ\text{C}$ , 48 h). HF was carefully removed under nitrogen, and samples were washed twice with  $\text{H}_2\text{O}$  to ensure complete evaporation of HF. SEC on a column (120 $\times$ 1.5 cm) of Biogel P-2 in pyridinium acetate (50 mM, pH 4.7) was conducted to separate monomers differing in size. A refractive index (Knauer) detector was used for detection of the monomers.

### Analytical chemistry

Single-component analysis of the EC TA was performed by GLC-MS after methanolysis (0.5 M HCl/MeOH,  $85^\circ\text{C}$ , 45 min) and acetylation (acetic anhydride and pyridine 1:1, by vol,  $85^\circ\text{C}$ , 7 min). For GLC investigations a Hewlett-Packard 5880 gas chromatograph equipped with a SPB-5 capillary column (30 m $\times$ 250  $\mu\text{m}$ , film thickness 0.25  $\mu\text{m}$ ) was used. A Hewlett-Packard 5989 instrument equipped with a HP-5MS capillary column (30 m $\times$ 250  $\mu\text{m}$ , film thickness 0.25  $\mu\text{m}$ ) was used for GLC-MS. Sugar analysis was carried out with a temperature program of  $120^\circ\text{C}$  (3 min),  $120$ – $260^\circ\text{C}$  at  $3^\circ\text{C}/\text{min}$ , then 10 min at  $260^\circ\text{C}$ . For fatty acid analysis, a temperature program of  $120^\circ\text{C}$  (3 min),  $120$ – $320^\circ\text{C}$  at  $5^\circ\text{C}/\text{min}$ , then 10 min at  $320^\circ\text{C}$  was run.

Sugars were identified as alditol acetates after hydrolysis (0.1 M HCl,  $100^\circ\text{C}$ , 48 h), reduction (pH 8,  $\text{NaBH}_4$ , 16 h, in the dark), and acetylation (acetic anhydride and pyridine 1:1, by vol,  $85^\circ\text{C}$ , 7 min) (Sawardeker and Sloneker, 1965). The absolute configurations of the neutral and amino sugars were determined by comparing the GLC retention times after butanolysis [2 M HCl/(S)-2-butanol,  $85^\circ\text{C}$ , 2 h] and acetylation as described above, in analogy to the authentic D-standards (Gerwig et al., 1979).

## Mass spectrometry

EC TA samples were analyzed by ESI FT-ICR MS on an APEX Qe-Instrument (Bruker Daltonics, Billerica, MA, USA) equipped with a 7-Tesla magnet and a dual Apollo ion source. Mass spectra were recorded in the negative ion mode under standard instrumental parameters. The sample (~10 ng/μl) was suspended in an ESI spray solution consisting of propan-2-ol, water, and triethylamine (50:50:0.001, by vol) and were sprayed at a flow rate of 2 μl/min. Capillary entrance voltage was set to 3.8 kV, and drying gas temperature to 180°C. Mass spectra were charge deconvoluted and all masses given refer to the monoisotopic peaks of the neutral molecules.

## NMR spectroscopy

For structural elucidation of the EC TA, NMR experiments were carried out on a Varian INOVA 600 MHz (<sup>1</sup>H) spectrometer with 3 mm gradient probe at 25°C in D<sub>2</sub>O, with acetone as internal reference (δ<sub>H</sub> 2.225 and δ<sub>C</sub> 31.45) using standard pulse sequences DQF-COSY, TOCSY, ROESY, HSQC, and HMBC. Spectra on fractions isolated after HF degradation were recorded at 700 MHz in D<sub>2</sub>O and 27°C with a Bruker DRX Avance III 700 MHz spectrometer (operating frequencies of 700.75 MHz for <sup>1</sup>H NMR, 176.2 MHz for <sup>13</sup>C NMR, and 283.67 for <sup>31</sup>P NMR). Chemical shifts were referenced to external acetone (δ<sub>H</sub> 2.225, δ<sub>C</sub> 31.45) and external 85% phosphoric acid (δ<sub>P</sub> 0.0) (Hanuszkiewicz et al., 2008a).

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