Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 340 (2005) 2290–2296
Carbohydrate RESEARCH

The secondary cell wall polymer of *Geobacillus tepidamans* GS5-97^T: structure of different glycoforms

Christian Steindl,[a] Christina Schäffer,[b] Vilko Smrečki,[c] Paul Messner[b] and Norbert Müller[a,*]

[a]*Institut für Organische Chemie, Johannes-Kepler-Universität Linz, Altenbergerstr. 69, A-4040 Linz, Austria*
[b]*Zentrum für NanoBiotechnologie, Universität für Bodenkultur Wien, Gregor-Mendel-Str. 33, A-1180 Wien, Austria*
[c]*NMR Center, Rudjer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia*

Received 23 May 2005; received in revised form 28 June 2005; accepted 12 July 2005
Available online 10 August 2005

Abstract—Nuclear magnetic resonance spectroscopic studies of the strain-specific secondary cell wall polymer (SCWP) of the Gram-positive, moderately thermophilic organism *Geobacillus tepidamans* GS5-97^T reveal two glycoforms consisting of identical tetrasaccharide repeating units with different chemical modifications of the amide moieties. On the basis of sugar analyses along with 1D and 2D ^{1}H, ^{13}C, ^{15}N, and ^{31}P NMR spectroscopy at natural isotope abundance, the basic backbone structure of the SCWP was established to be [β-D-Man*p*-2,3-diNAcANH_2-(1→6)-α-D-Glc*p*-(1→4)-β-D-Man*p*-2,3-diNAcANH_2-(1→3)-α-D-Glc*p*NAc-(1→]$_6$-(1→O)-PO_2-(O→6)-MurNAc-, with modifications of the amide groups. In one glycoform, all β-D-Man*p*-2,3-diNAcANH_2 (2,3-diacetamido-2,3-dideoxy-β-D-mannopyranuronamide, Man*p*ANH_2) residues are substituted with two acetyl groups (glycoform I) at the amide group at C-6; in the other glycoform (glycoform II), only one proton of this amide group is substituted by an acetyl group. The ratio between both the glycoforms approximates 1:1.

Keywords: *Geobacillus tepidamans* GS5-97^T; Secondary cell wall polymer (SCWP); Bacterial polysaccharide structure; Glycoforms; Chemical modification

1. Introduction

Geobacillus tepidamans strain GS5-97^T is the type strain of a recently described novel species among moderately thermophilic bacilli.[1] Together with other strains, it was isolated from extraction-juice samples of an Austrian beet sugar factory. *G. tepidamans* GS5-97^T is capable of synthesizing glycosylated surface layer (S-layer) proteins constituting the outermost cell envelope layer of the bacterium.[2,3] The S-layer glycoprotein glycan chains of *G. tepidamans* GS5-97^T are assembled from [→3)-α-L-rhamnopyranose-(1→2)-α-D-fucopyranose-(1→]$_{n\sim20}$-disaccharide repeating units.[4] The glycoprotein is proposed to be attached to the peptidoglycan layer (PG) by saccharide chains, designated as secondary cell wall polymer (SCWP). Such saccharide structures are well known from many other Gram-positive bacteria.[5–12] The term 'secondary wall polymers' has been introduced by the first generation of researchers in the field of Gram-positive cell walls,[13] which has also been reviewed most recently.[14] The comparison of structurally investigated SCWPs from Gram-positive S-layer-covered organisms revealed that they are, with one exception known so far, built of distinct repeating units, with variations within a given species arising exclusively from differences in glycan chain lengths, thereby possibly reflecting a repeating unit-wise SCWP biosynthesis mechanism.[9,14,15] Here, we report for the first time on different glycoforms of a naturally occurring SCWP. The SCWP of *G. tepidamans* GS5-97^T possesses the same repeating unit backbone structure as the SCWP of *Geobacillus stearothermophilus* NRS 2004/3a,[9] with

* Corresponding author. Tel.: +43 732 2468 8746; fax: +43 732 2468 8747; e-mail: norbert.mueller@jku.at

0008-6215/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2005.07.005

modifications of the carboxyl groups of the mannosaminuronic acid residues.

2. Results and discussion

2.1. Isolation and chemical characterization of the SCWP glycoforms

From about 600 mg of PG preparation of *G. tepidamans* GS5-97^T, two major SCWP fractions, designated glycoform I and II, were isolated. While the two glycoforms co-eluted in the void volume of a Sephadex G-50 column, where the majority of SCWP-free PG break-down products were removed, their separation based on differences in the molecular masses was subsequently accomplished by a combination of Bio-Gel P-30 and P-60 chromatography. Twenty eight milligrams of the high-molecular mass glycoform I obtained after Bio-Gel P-60 chromatography finally yielded 6.3 mg of material after RP(C18)-HPLC.[9] This material was subjected to NMR analysis. From the more acidic glycoform II (lower molecular mass), 5.5 mg of a pure sample was obtained after re-chromatography on the Bio-Gel P-30 column. The summarized yields of glycoforms I and II indicated that the SCWP constitutes approximately 2% of *G. tepidamans* GS5-97^T PG. Carbohydrate analysis by HPAED-PED showed identical carbohydrate compositions of the glycoforms, with Glc and GlcNAc being present in an equimolar ratio. The content of the PG amino acids glutamic acid, alanine, histidine, diaminopimelic acid, and muramic acid varied slightly. Their molar ratios were 1.1:0.8:1.2:1.0:2.0 in glycoform I, and 1.0:0.9:1.1:1.0:1.0 in glycoform II, respectively, with diaminopimelic acid being set to the value of 1.0.

2.2. NMR spectroscopic studies and structure of the SCWP glycan forms

For the interpretation of NMR data, capital bold letters denote individual monosaccharide residues, following their sequence in the proposed oligosaccharide structures (Fig. 1). In the anomeric region of the $^{13}C/^{1}H$-HSQC spectra of both samples, four strong (**A**–**D**) and three medium-intensity cross-peaks (**A′**, **B′**, **D′**) could be distinguished. The integral ratios of these peaks were 1:1:6:5:5:5:1 (**A′**:**B′**:**C**:**D**:**A**:**B**:**D′**), which was in accordance with the integrals of the corresponding regions in the 1D ^{1}H spectra. This pattern was explained by an oligosaccharide sequence consisting of a basic tetrasaccharide building block, repeated six times. Only the first two monosaccharide residues at the reducing end and a single residue at the non-reducing end gave non-degenerate NMR signals. All spectroscopic and chemical evidence is consistent with this interpretation, as will be described below.

One-bond C–H coupling constants revealed the configuration at the anomeric C-1 atoms to be α for units **A′**, **A**, and **C** ($^{1}J_{C,H} \sim 172$ Hz) and β for **B′**, **B**, **D**, and **D′** ($^{1}J_{C,H} \sim 162$ Hz). Starting from these anomeric positions, an almost complete assignment of all backbone protons and carbons was obtained (see Table 1) by combining intra-glycose information from 2QF-COSY- and $^{13}C/^{1}H$-HSQC-TOCSY spectra with inter-glycose connectivities obtained mostly from HMBC spectra. Figure 2 illustrates the types of correlations leading to these

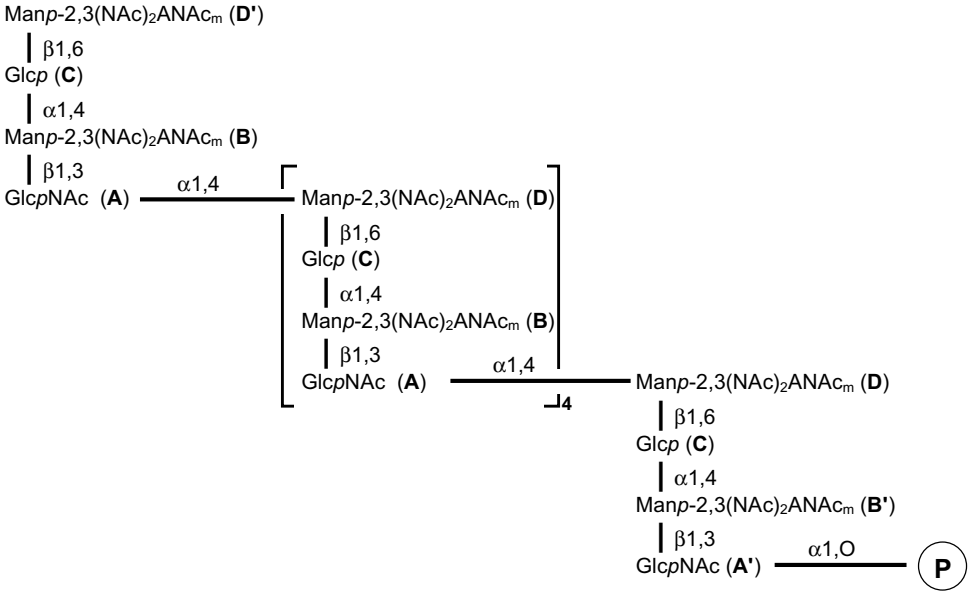

Figure 1. Structure of the SCWP isolated from the PG of *Geobacillus tepidamans* GS5-97^T occurring in two distinct glycoforms. Glycoform I, $m = 2$; glycoform II, $m = 1$. The bold capital letters designating the sugar residues are used to indicate NMR assignments throughout this text.

Table 1. ^{13}C, ^{15}N (upper lines), and ^{1}H (lower lines) NMR chemical shift (ppm) assignments for the SCWP glycoforms I and II of *G. tepidamans* GS5-97^{T} (n.a.: not assignable)

Glycose residue	C/H-atom						2-*N*-Acetyl			3-*N*-Acetyl		
	1	2	3	4	5	6	CO	CH_3	NH	CO	CH_3	NH
Glycoform I												
α-D-Glc*p*NAc (**A′**)	95.65	52.82	82.30	n.a.	72.00	n.a.	n.a.	n.a.	n.a.	—	—	—
	5.49	4.09	3.99	3.64	4.03	3.68/3.91	n.a.	n.a.	n.a.	—	—	—
α-D-Glc*p*NAc (**A**)	98.46	53.34	82.30	69.52	73.75	61.39	175.26	23.52	121.33	—	—	—
	5.16	3.99	3.85	3.64	3.65	3.81/3.85	—	2.05	8.26	—	—	—
β-D-Man*p*-2,3-diNAcANH(Ac) (**B′**)	100.92	52.95	54.84	73.03	78.23	n.a.	176.04	23.54	113.25	n.a.	n.a.	n.a.
	5.12	4.39	4.18	3.95	4.12	—	—	2.11	8.14	n.a.	n.a.	n.a.
β-D-Man*p*-2,3-diNAcANH(Ac) (**B**)	101.13	52.91	54.40	73.06	77.86	n.a.	176.04	23.53	113.08	175.88	23.50	122.65
	5.04	4.38	4.37	3.97	4.17	—	—	2.10	8.09	—	1.99	8.39
α-D-Glc*p* (**C**)	100.42	70.34	73.92	72.65	72.69	69.74	—	—	—	—	—	—
	5.13	3.43	3.61	3.43	3.69	3.90/4.06	—	—	—	—	—	—
β-D-Man*p*-2,3-diNAcANH(Ac) (**D**)	101.33	52.36	54.52	72.00	77.83	n.a.	176.76	23.56	114.46	175.23	23.38	122.11
	4.97	4.46	4.38	4.05	4.18	—	—	2.13	8.14	—	1.94	8.41
β-D-Man*p*-2,3-diNAcANH(Ac) (**D′**)	101.40	52.22	54.83	67.46	78.18	n.a.	176.04	23.53	113.08	n.a.	n.a.	127.02
	4.98	4.57	4.16	3.77	4.11	—	—	2.10	8.09	—	n.a.	8.20
α-MurNAc	91.78	55.16	77.92	77.61	77.96	72.98	n.a.	n.a.	n.a.			
	5.29	3.89	3.76	3.90	3.79	4.18/4.38	n.a.	n.a.	n.a.			
β-MurNAc	101.96	57.51	77.59	75.21	75.15	71.96	n.a.	n.a.	n.a.			
	4.59	3.79	3.47	3.58	3.61	4.18/4.38	n.a.	n.a.	n.a.			
Glycoform II												
α-D-Glc*p*NAc (**A′**)	95.60	53.90	82.54	n.a.	n.a.	n.a.	175.74	23.45	120.74	—	—	—
	5.47	4.06	3.95	3.63	3.93	3.60/3.88	—	2.07	8.22	—	—	—
α-D-Glc*p*NAc (**A**)	98.30	53.50	82.67	69.65	73.14	61.48	175.25	23.43	121.72	—	—	—
	5.12	3.94	3.87	3.57	3.78	3.81/3.82	—	2.01	8.27	—	—	—
β-D-Man*p*-2,3-diNAcAN(Ac)$_2$ (**B′**)	101.31	53.21	54.80	72.35	79.95	n.a.	176.09	23.46	113.70	n.a.	n.a.	n.a.
	5.04	4.34	4.34	3.94	3.95	—	—	2.06	8.21	—	n.a.	n.a.
β-D-Man*p*-2,3-diNAcAN(Ac)$_2$ (**B**)	101.37	53.20	54.80	72.36	79.95	n.a.	176.09	23.46	113.70	175.71	23.52	123.33
	4.98	4.30	4.34	3.94	3.95	—	—	2.06	8.21	—	1.95	8.38
α-D-Glc*p* (**C**)	99.75	72.83	74.07	70.16	72.36	69.22	—	—	—	—	—	—
	5.13	3.37	3.63	3.43	3.77	3.92/4.01	—	—	—	—	—	—
β-D-Man*p*-2,3-diNAcAN(Ac)$_2$ (**D**)	101.07	52.90	54.82	72.36	79.90	n.a.	176.64	23.51	115.00	175.25	23.34	122.63
	4.91	4.39	4.32	3.94	3.95	—	—	2.09	8.24	—	1.90	8.38
β-D-Man*p*-2,3-diNAcAN(Ac)$_2$ (**D′**)	101.07	52.45	55.00	68.14	79.80	n.a.	176.58	23.51	n.a.	n.a.	n.a.	n.a.
	4.91	4.50	4.09	3.68	3.86	—	—	2.09	n.a.	—	n.a.	n.a.
α-MurNAc	91.80	n.a.	n.a.	n.a.	n.a.	72.98	n.a.	n.a.	n.a.			
	5.25	3.89	3.79	3.87	n.a.	4.8/4.38	n.a.	n.a.	n.a.			
β-MurNAc	101.62	n.a.	n.a.	n.a.	n.a.	71.96	n.a.	n.a.	n.a.			
	4.59	3.76	3.60	3.47	n.a.	4.18/4.38	n.a.	n.a.	n.a.			

Bold capital letters refer to Figure 1. The acetate groups at C-6 of mannosaminuronamides (**B**, **B′**, **D**, and **D′**) were not assignable (see text).

assignments for the two units **A** and **C** and the **B**–**A**(1→3) and **D**–**C**(1→6) glycosidic linkages. Several HMBC spectra optimized for different ^{13}C–^{1}H coupling constants (from 3 to 10 Hz) were required to obtain all inter-glycose correlations. Units **A′** and **A** were identified as α-GlcNAc residues, showing typical chemical shifts for ^{1}H and ^{13}C and $^{3}J_{2,3}$ coupling constants of about 10 Hz.[18] A typical deshielding of ^{1}H (~0.2 ppm) and ^{13}C (~12 ppm) at position 3 indicated the position of the glycosidic linkage. The characteristically separated chemical shift of H-1 of unit **A′** (5.49 ppm in glycoform I; 5.47 ppm in glycoform II) was in accordance with previous work[9,16,17] and is typical of the reducing end of a saccharide, linked to a PG via a phosphate-containing bridge.[16] The chemical shifts (Table 1) and the coupling constants of monomer unit **C** identified this sugar as an α-Glc residue, glycosylated at C-6, as indicated by deshielding of ^{1}H (~0.2 ppm) and ^{13}C (~10 ppm) and the larger (~0.1 ppm) separation of the diastereotopic H-6 protons compared to unit **A** (see Fig. 3). The positions of the glycosidic linkages were validated by further $^{1}H/^{13}C$-HMBC and $^{1}H/^{1}H$-NOESY experiments.

Assignment of the remaining peaks corresponding to units **B**, **B′**, **D**, and **D′** was straightforward (Table 1). Structural interpretation revealed novel features not described so far in SCWPs.[14] These monomer units were 2,3-di-*N*-acetyl-2,3-dideoxy-aminosugars, as evidenced by their C-2 and C-3 ^{13}C chemical shifts of about 52–55 ppm. Chemical shift comparison together with $^{3}J_{2,3}$ coupling constants of about 4 Hz (due to overlap only the coupling constants of **B′**, **D**, and **D′** in glycoform I and **B**, **D**, and **D′** in glycoform II could be analyzed) indicated *manno*-configured sugars.[9] The linkage

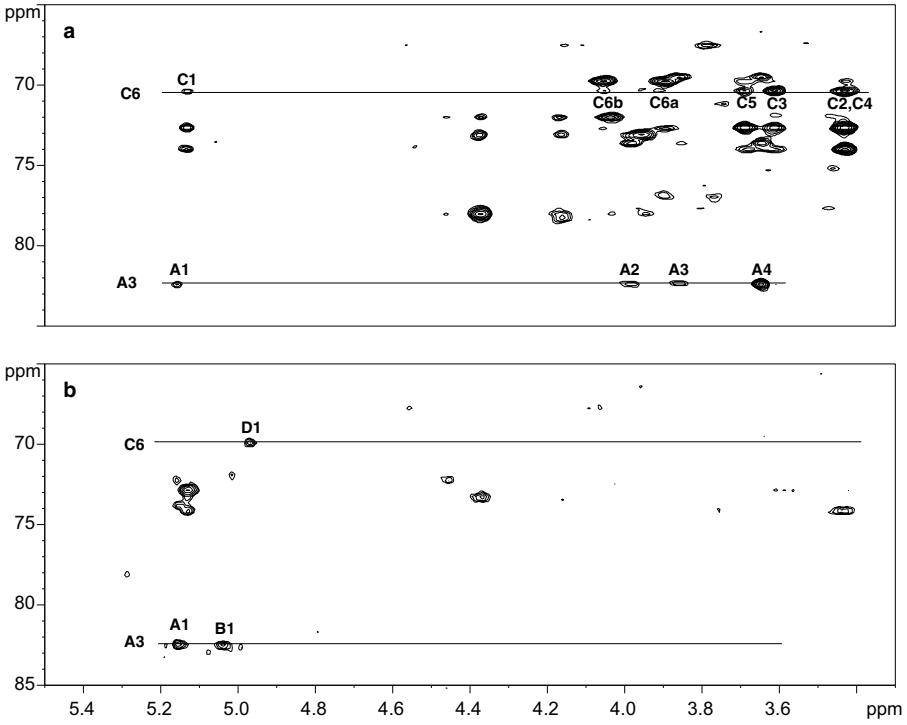

Figure 2. Absorptive $^{1}H/^{13}C$-HSQC-TOCSY ((a); 60 ms mixing time) and magnitude HMBC ((b); optimized for 10 Hz heteronuclear coupling constants) spectra of glycoform I of the SCWP from *Geobacillus tepidamans* GS5-97^{T}. In spectrum (a), intra-glycose C-6/H correlations for unit **C** and C-3/H correlations for unit **A** are indicated. In the HMBC spectrum (b), inter-glycose linkages are shown for the same carbon atoms. Bold capital letters refer to sugar residues as defined in Figure 1. The corresponding horizontal lines emphasize the complementary information in both spectra.

position of these residues is corroborated by the deshielding of H-4 ($\delta_{H\text{-}4} \sim 3.94$–4.05 ppm). There is no comparable deshielding effect at C-4 of unit **D′** ($\delta_{H\text{-}4} = 3.77$ ppm in glycoform I, 3.68 ppm in glycoform II), because this sugar is at the non-reducing end of the SCWP.

In HSQC spectra (Fig. 3), no signals for CH_2-6 were found for the *manno*-configured sugars. No unassigned C–H one-bond correlation peaks with amplitudes corresponding to one or more sugars of the 24 residues constituting the SCWP remained in the spectrum. From this and from the observed deshielding of C-5 (at ~80 ppm) and H-5 (at ~4 ppm), we conclude that these sugars are oxidized at position 6, indicating the presence of mannuronic acid derivatives.

To this point, the saccharide structure of the SCWP of GS5-97^{T} is reminiscent of that of *G. stearothermophilus* NRS 2004/3a.[9] However, in ^{13}C NMR and HMBC (at several coherence transfer times) spectra, no signals corresponding to carboxylate carbons were found. All carbonyl signals appeared within a narrow range of 175–177 ppm characteristic of amides and imides. This finding led to the conclusion that these uronic acid groups are amidated. Integration of the 1D-^{1}H spectra revealed significant differences in the acetyl proton regions (1.85–2.15 ppm) of the two glycoforms. In glycoform I, ~169 acetyl protons were found, compared to ~132 acetyl protons in glycoform II (Fig. 4). This difference of ~37 protons closely corresponds to two acetyl groups for each of the six tetrasaccharide repeating units. Taking into account a small number of overlapping amino acid signals in the same spectral region (as is evident from the small cross-peaks in Fig. 3) this corresponds to a total of 54 and 42 acetyl groups for glycoforms I and II, respectively.

Attempts to use ^{15}N–^{1}H correlation spectra to follow the connectivity of the acetyl groups to the sugar rings were only partially successful. Assignment of the ^{15}N signals (Table 1) of most 2-NAc and 3-NAc ^{15}N chemical shifts was achieved by ^{15}N-CH_3 correlation in HMBC spectra, relating ^{15}N chemical shifts to the CH_3-proton resonances, which had been assigned from the NOESY spectra. The additional *N*-acetyl methyl protons, clearly visible in the ^{1}H NMR spectra (Fig. 4), did not result in resolved cross-peaks in $^{1}H/^{15}N$ HMBC spectra of both glycoforms. Presumably, this is due to local conformational dynamics around the amide bonds.

$^{15}N/^{1}H$-HSQC spectra of the two glycoforms revealed no significant differences in the integral ratios for the 2- and 3-NH-cross-peaks of N-acetylglucosamines and N-acetylmannuronamides. The only possibility to explain these observations was the occurrence of different degrees of acetylation of the amide-N at C-6. The spectroscopic data together with the chemical

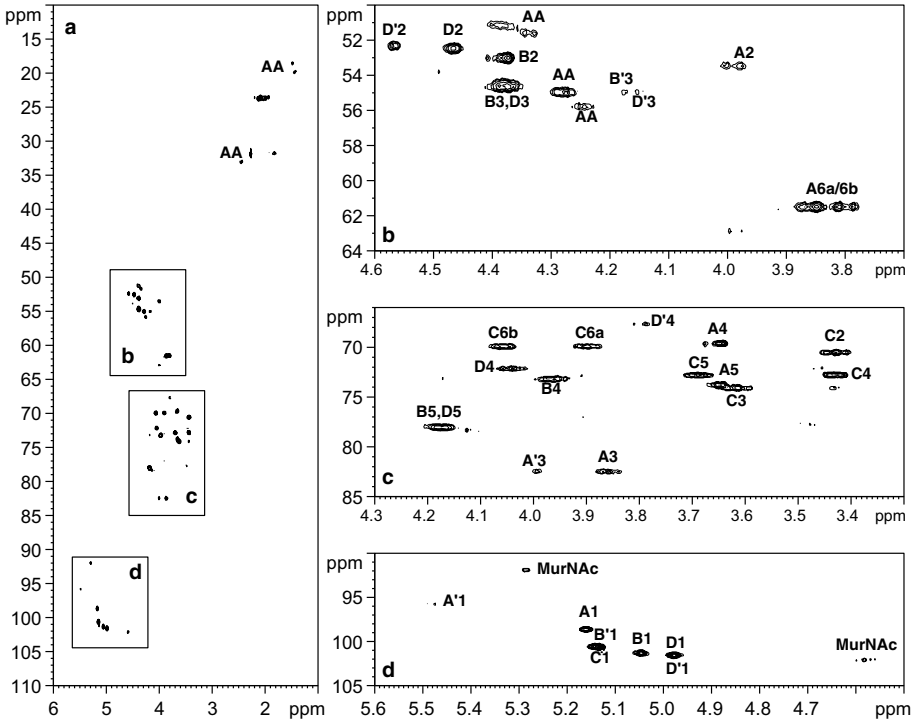

Figure 3. Overview (a) and expanded regions (b)–(d) of the absorptive, sensitivity improved $^{1}H/^{13}C$-HSQC spectrum of glycoform I of the SCWP from *Geobacillus tepidamans* GS5-97^{T}. The labels indicate the assignments using the one-letter code from Figure 1 and the ring positions by numbers. The resonances clustered around 24 ppm (^{13}C)/2 ppm (^{1}H) are due to the *N*-acetyl groups. Peaks originating from amino acids of PG are labeled **AA**. All resonances from N-acetylated positions in amino sugars are present in the region b (expanded in (b)). The other non-anomeric positions (with the exception of CH_2-6 in Glc*p*NAc) are present in region c (expanded in (c)), while the anomeric signals are well separated in region d (expanded in (d)). It should be noted that no carbohydrate signals remained unassigned at the peak amplitude levels corresponding to the unique monomer units.

information of the two glycoforms led to the conclusion that the amide nitrogens of all mannosaminuronamide residues were di-acetylated in glycoform I and mono-acetylated in glycoform II. To summarize, we propose the following structure of the mannosaminuronamide residues (**B**, **B**′, **D**, and **D**′): glycoform I contains 6-*N*,*N*-diacetyl-2,3-diacetamido-2,3-dideoxy-β-D-mannuronamide, while glycoform II contains 6-*N*-acetyl-2,3-diacetamido-2,3-dideoxy-β-D-mannuronamide. This finding is corroborated by the higher acidity of the mono-acetylated glycoform II due to fast exchanging imide protons (the pH of an aqueous solutions of glycoform I was 4.6; while that of glycoform II was pH 2.8). The impossibility to detect any correlation between H-5 and the carboxamide-C-6 was attributed to an unfavorable combination of local dynamics and long-range coupling constants in this imide group.

The glycosidic linkages were delineated using $^{13}C/^{1}H$-HMBC and $^{1}H/^{1}H$-NOESY. The linkage between units **A**′ and **B**′ was proven by NOE cross-peaks between H-1 of **B**′ and H-2 of **A**′. All other glycosidic linkages (**B**-H-1→**A**-C-3, **C**-H-1→**B**′-C-4, **C**-H-1→**B**-C-4, **D**-H-1→**C**-C-6, **D**′-H-1→**C**-C-6, and **A**-H-1→**D**-C-4) were observed in $^{13}C/^{1}H$-HMBC spectra and confirmed by NOE cross-peaks. The most important cross-peaks for structure elucidation were **B**′-H-1→**A**′-H-2, **B**-H-1→**A**-H-3, **C**-H-1→**B**′-H-4/**B**-H-4, **A**-H-1→**D**-H-4, **D**-H-1→**C**-H-6, and **D**′-H-1→**C**-H-6.

The linkage of the reducing end α-D-GlcpNAc (**A**′) to muramic acid of the PG moiety via a phosphodiester bond was derived from indirect evidence. For muramic acid, only the major signals are listed in Table 1, since heterogeneity of the PG moiety, arising from varying degrees of degradation during sample preparation leads to considerable signal splitting and assignment ambiguity.[9]

The ^{31}P NMR spectrum showed one broad resonance at 0.64 ppm (~45 Hz line width), which is characteristic of a phosphodiester bond. $^{1}H\{^{31}P\}$ decoupling difference correlation spectra as used earlier[14] did not allow unequivocal discrimination between subtraction artifacts and true signals (due to the inherently lower sensitivity of the non-cryogenic NMR probe used for ^{31}P experiments). However, from the observed ^{1}H and ^{13}C chemical shifts, a 1→6 linkage between muramic acid and a phosphate group is most likely. No evidence for a pyrophosphate was found. Approximately, equal amounts of terminal α- and β-configured muramic acid residues were present in the PG. This part of the structure was not further analyzed.

In conclusion, two glycoforms of a SCWP were isolated from a PG preparation of *G. tepidamans* GS5-97^{T}. The glycan moieties of both the glycoforms are

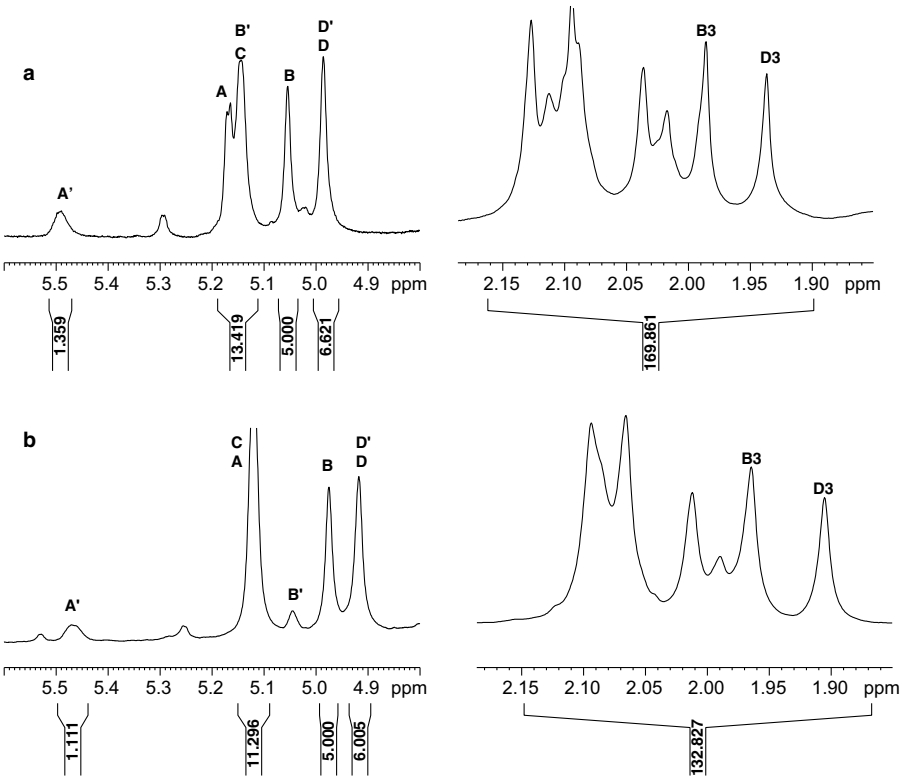

Figure 4. Partial proton NMR spectra of glycoform I (a) and glycoform II (4b) of the SCWP from *Geobacillus tepidamans* GS5-97^T. The characteristic differences in the anomeric and methyl regions as discussed in the text are evident from the peak patterns and the numeric integrals. The peak labels indicate the assignments using the one-letter code from Figure 1. The integrals were normalized on the anomeric signal of unit **B** (the drawing amplitudes of the low and high frequency parts of the spectra are scaled differently to fit to the available space).

linked at the reducing end to muramic acid residues of the PG sacculus of the bacterial cell wall via a phosphodiester bond. The 24 sugar residues of the SCWP constitute six linear tetrasaccharide repeating units, which contain alternating α-*gluco*- and β-*manno*-configured sugars. The *manno*-configured sugars are uniformly substituted over the entire SCWP, but characteristically different in the two glycoforms. To our knowledge this is the first report on such SCWP modifications; glycoform I contains 6-*N*,*N*-diacetyl-2,3-diacetamido-2,3-dideoxy-β-D-mannuronamide, and glycoform II contains 6-*N*-acetyl-2,3-diacetamido-2,3-dideoxy-β-D-mannuronamide residues.

3. Experimental

3.1. Bacterial cultivation and general methods

Fed-batch cultivation of *G. tepidamans* GS5-97^T was carried out in a Braun Biostat C 15-l fermenter (B. Braun, Melsungen, Germany) at 55 °C at a constant pH value of 7.0, with a dilution rate of 0.3 h^{-1} and an oxygen saturation of 30%.[1] Cells were separated from the culture broth by centrifugation at 16,000*g* at 4 °C, and the cell pellet was stored at −20 °C.

Analytical methods for carbohydrate analysis, amino acid analysis, and determination of phosphate were described previously.[18–20]

3.2. Isolation of SCWP

For isolation of SCWP, 125 g of frozen cells were thawed in distilled water, re-suspended to homogeneity and broken by mild sonication in an ice bath (Branson sonicator; output 70%, 3 cycles, 5 min each). Cell pellets were collected by centrifugation at 48,000*g* (4 °C) and washed with 1 M NaCl. Degradation of protein, re-N-acetylation, and lysozyme digestion were performed according to the published procedures.[16] The soluble fraction from the lysozyme digest was applied to subsequent columns of Sephadex G-50 (Amersham Pharmacia Biotech) and Bio-Gel P-30 (Bio-Rad) as described previously.[9] The high molecular-mass pool after P-30 chromatography was further fractionated on Bio-Gel P-60 (Bio-Rad) and the carbohydrate-containing pool was finally purified by C-18 reversed-phase HPLC (Nucleosil 120-3C18; Machery & Nagel) in a water/CH_3CN gradient,[9] yielding glycoform I. The carbohydrate-containing material eluting within the fractionation range of the P-30 column was re-chromatographed on the same column and yielded glycoform II. The two glycoforms

were subsequently lyophilized and investigated by NMR spectroscopy.

3.3. NMR spectroscopic analysis

All ^{1}H, ^{13}C, and ^{15}N NMR spectra were obtained at a sample temperature of 313.4 K on a Bruker DRX 500 MHz instrument equipped with a triple resonance inverse detection (TXI; ^{1}H, ^{13}C, ^{15}N; inner coil tuned for ^{1}H) cryogenically cooled probe (radio frequency coil temperature 30 K) with a built-in z-gradient coil. Typical 90° pulse lengths were 9 μs for ^{1}H, 13 μs for ^{13}C, and 45 μs for ^{15}N. Standard gradient enhanced pulse programs supplied in the instruments' manufacturer's library were used. Lyophilized glycoform I (5.5 mg) and glycoform II (4 mg), respectively, were dissolved in 450 μL of 99.96% $^{2}H_2O$, except for the $^{15}N/^{1}H$ experiments, where a mixture of 90% H_2O and 10% $^{2}H_2O$ was used as solvent. Spectra were referenced with respect to the methyl signal of external sodium trimethylsilylpropionate (TSP) at 0.0 ppm. In the ^{1}H observation dimension, 4 K data points were acquired at a spectral width of 5 kHz, with the carrier frequency set to the residual water signal. In the indirect carbon dimension, 512 K data points or 2 K data points (for high resolution phase sensitive HSQC and magnitude-mode HMBC spectra) at spectral widths of 100 ppm (HSQC-type experiments) or 190 ppm (HMBC) were used. In HSQC-TOCSY experiments a mixing time of 60 ms was used with a pulsed (MLEV) spin lock field of 3.13 kHz. GARP decoupling was employed for ^{13}C and ^{15}N during proton detection, where applicable. NOESY mixing times were 100 ms. Up to 128 transients were recorded per evolution time increment.

^{31}P spectra were recorded on a Bruker Avance AV 600 MHz spectrometer at the same sample temperature using a BBO (BB, ^{1}H; inner coil tuned for ^{31}P) probe with broadband proton decoupling (WALTZ). 85% H_3PO_4 served as external reference.

Chemical shifts are given in ppm with precision of $\pm0.12/\pm0.2/\pm0.02/\pm0.2$ ppm for $^{13}C/^{15}N/^{1}H/^{31}P$ spectra, respectively. Coupling constants are given in Hertz, with precision of 0.1/0.5 Hz for ^{1}H–$^{1}H/^{1}H$–^{13}C couplings. All experimental data were zero-filled to double or to quadruple, in case of the ^{13}C dimension of HMBC, the number of experimental points. Linear prediction was used instead of zero-filling for the processing of the indirect dimensions in heteronuclear experiments prior to Fourier transformation. All spectra were baseline-corrected using automatic 5th degree polynomial baseline correction. The software XWINNMR version 3.5 (Bruker BioSpin) was used for all acquisition and processing.

Acknowledgements

We thank Sonja Zayni and Andrea Scheberl for excellent technical assistance. The work was supported by the Austrian Science Fund, projects P15612-B07 and P15840-B10 (to P.M.) and P15380 (to N.M.) and the Austrian-Croatian joint research project 91102/03-06 (ÖAD-Project 13/04, to V.S. and N.M.).

References

1. Schäffer, C.; Franck, W. L.; Scheberl, A.; Kosma, P.; McDermott, T. R.; Messner, P. *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 2361–2368.
2. Sleytr, U. B.; Messner, P. In *Desk Encyclopedia of Microbiology*; Schaechter, M., Ed.; Elsevier Science: San Diego, 2003, pp 286–293.
3. Schäffer, C.; Messner, P. *Glycobiology* **2004**, *14*, 31R–42R.
4. Kählig, H.; Kolarich, D.; Zayni, S.; Scheberl, A.; Kosma, P.; Schäffer, C.; Messner, P. *J. Biol. Chem.* **2005**, *280*, 20292–20299.
5. Araki, Y.; Ito, E. *CRC Crit. Rev. Microbiol.* **1989**, *17*, 121–135.
6. Archibald, A. R.; Hancock, I. C.; Harwood, C. R. In *Bacillus subtilis and Other Gram-Positive Bacteria*; Sonenshein, A. L., Hoch, J. A., Losick, R., Eds.; American Society for Microbiology Press: Washington, DC, 1993, pp 381–410.
7. Naumova, I. B.; Shashkov, A. S. *Biochemistry (Moscow)* **1997**, *62*, 809–840.
8. Ries, W.; Hotzy, C.; Schocher, I.; Sleytr, U. B.; Sára, M. *J. Bacteriol.* **1997**, *179*, 3892–3898.
9. Schäffer, C.; Kählig, H.; Christian, R.; Schulz, G.; Zayni, S.; Messner, P. *Microbiology* **1999**, *145*, 1575–1583.
10. Mesnage, S.; Fontaine, T.; Mignot, T.; Delepierre, M.; Mock, M.; Fouet, A. *EMBO J.* **2000**, *19*, 4473–4484.
11. Sára, M. *Trends Microbiol.* **2001**, *9*, 47–50.
12. Cava, F.; de Pedro, M. A.; Schwarz, H.; Henne, A.; Berenguer, J. *J. Mol. Microbiol.* **2004**, *52*, 677–690.
13. Ward, J. B. *Microbiol. Rev.* **1981**, *45*, 211–243.
14. Schäffer, C.; Messner, P. *Microbiology* **2005**, *151*, 643–651.
15. Ilk, N.; Kosma, P.; Egelseer, E. M.; Mayer, H. F.; Sleytr, U. B.; Sára, M. *J. Bacteriol.* **1999**, *181*, 7643–7646.
16. Schäffer, C.; Müller, N.; Mandal, P. K.; Christian, R.; Zayni, S.; Messner, P. *Glycoconjugate J.* **2000**, *17*, 681–690.
17. Steindl, C.; Schäffer, C.; Wugeditsch, T.; Graninger, M.; Matecko, I.; Müller, N.; Messner, P. *Biochem. J.* **2002**, *368*, 483–494.
18. Altman, E.; Schäffer, C.; Brisson, J.-R.; Messner, P. *Eur. J. Biochem.* **1995**, *229*, 308–315.
19. Winzler, R. J. *Methods Biochem. Anal.* **1955**, *11*, 279–311.
20. Ames, B. N. *Methods Enzymol.* **1966**, *VIII*, 115–118.