The diacetamidodideoxyuronic-acid-containing glycan chain of *Bacillus stearothermophilus* NRS 2004/3a represents the secondary cell-wall polymer of wild-type *B. stearothermophilus* strains

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The diacetamidodideoxymannuronic-acid-containing glycan of *Bacillus stearothermophilus* NRS 2004/3a with the repeating unit structure [$\rightarrow 4$)- β -D-ManpA2,3(NAc)₂-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 4)- β -D-ManpA2,3(NAc)₂-(1 $\rightarrow$ 3)- α -D-GlcpNAc-(1 $\rightarrow$], was examined to identify its linkage to the bacterial cell wall. In a previous paper it was suggested that this glycan is covalently linked to the surface layer (S-layer) glycoprotein of that organism. By improved chromatographic techniques (gel permeation over Sephacryl S-1000 SF; C4 reversed-phase HPLC) the diacetamidodideoxyuronic-acid-containing material was completely separated from the S-layer glycoprotein. This implicates only low, if any, specific affinity between these cell-wall components. To obtain sufficient amounts for the chemical characterization of its linkage region, the identical diacetamidodideoxyuronic-acid-containing material was isolated from sonicated cells of that organism by a purification procedure different to that for preparation of S-layers. This method allowed collection of the intact molecule including its linkage region. From the combined results of the chemical characterization and 600 MHz NMR spectroscopy it is proposed that the diacetamidodideoxyuronic-acid-containing glycan chain, consisting of approximately six tetrasaccharide repeating units, is directly linked via a pyrophosphate bridge to carbon 6 of muramic acid residues of the peptidoglycan sacculus. About 20–25% of the muramic acid residues are substituted with these polysaccharide chains. Thus, the diacetamidodideoxyuronic-acid-containing glycan represents a secondary cell-wall polymer of *B. stearothermophilus* NRS 2004/3a.

Keywords: *Bacillus stearothermophilus*, secondary cell-wall polymer, linkage region, peptidoglycan, surface layer (S-layer)

INTRODUCTION

The surface layer (S-layer) of *Bacillus stearothermophilus* NRS 2004/3a is composed of glycoprotein subunits. The repeating unit structure of the glycan chains was determined to be [$\rightarrow 2$)- α -L-Rhap-(1 $\rightarrow$ 2)- α -

L-Rhap-(1 $\rightarrow$ 3)- β -L-Rhap-(1 $\rightarrow$)_{n-50} (Christian *et al.*, 1986). In addition to the N-glycosidically linked S-layer glycoprotein glycans (Messner & Sleytr, 1988a) this organism possesses cell-wall-associated carbohydrate chains. Their repeating unit structure [$\rightarrow 4$)- β -D-ManpA2,3(NAc)₂-(1 $\rightarrow$ 6)- α -D-Glcp-(1 $\rightarrow$ 4)-

Abbreviations: DFQ-COSY, double quantum filtered correlation spectroscopy; GARP, globally optimized alternating-phase rectangular pulses; ge, gradient-enhanced; HMBC, hetero multiple bond correlation spectroscopy; HPAEC-PED, high-performance anion-exchange chromatography with pulsed electrochemical detection; HSQC, heteronuclear single quantum correlation spectroscopy; ManpA2,3(NAc)₂, 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid; MurNAc, N-acetyl-D-muramic acid; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reversed-phase high-performance liquid chromatography; S-layer, surface layer; TFA, trifluoroacetic acid; TOCSY, total correlated spectroscopy; TPPI, time proportional phase incrementation.

β -D-ManpA2,3(NAc)₂-(1 → 3)- α -D-GlcpNAc-(1 →) was elucidated by 250 MHz NMR spectroscopy (Messner *et al.*, 1987). The perception that more than one different glycan chain can be covalently linked to an S-layer protein monomer (Lechner & Wieland, 1989) had previously led to the assumption that these carbohydrate chains might be covalently linked to the S-layer protein. However, several attempts to identify a putative carbohydrate-protein linkage region were not successful.

In previous work, the S-layer material was isolated by a standard procedure (Messner & Sleytr, 1988b) followed by thorough proteolytic digestion of the purified S-layer glycoprotein with Pronase E. Gel filtration of the degradation products through a Bio-Gel P-30 column had completely separated the rhamnan-containing oligosaccharide fraction from the ManpA2,3(NAc)₂-containing glycan chain. Neither in the NMR spectra nor in the corresponding amino acid analyses was there an indication for the presence of a covalent linkage of the ManpA2,3(NAc)₂ (2,3-diacetamido-2,3-dideoxy-D-mannuronic acid)-containing glycan chain to the S-layer protein (Messner *et al.*, 1987). Only amino acids not involved in conventional N- and O-glycosidic linkages had been identified.

In this present study, we have defined the linkage type of the ManpA2,3(NAc)₂-containing glycan to the cell wall of *B. stearothermophilus* NRS 2004/3a and investigated whether there is affinity between the S-layer glycoprotein and the coeluting ManpA2,3(NAc)₂-containing glycan. Since only minor amounts of this material are present in the S-layer preparation, for the structural work on the intact molecule it was isolated from a peptidoglycan preparation of that strain.

METHODS

Growth of the bacteria. *B. stearothermophilus* NRS 2004/3a (Messner *et al.*, 1984) was grown in continuous culture on modified SVIII medium (0.5% peptone, 0.5% meat extract, 0.5% yeast extract, 0.2% glucose, 0.13% K₂HPO₄, 0.01% MgSO₄, pH ~6.9) at 60 °C with an oxygen partial pressure of 10% (v/v) and a dilution rate of 0.2 h⁻¹ in a 15 l Biostat C fermenter (B. Braun). The pH of the culture was kept constant at a value of pH 7.0 ± 0.25 by addition of 2 M sulfuric acid. Cells were separated from culture broth by continuous centrifugation (Sepatech 17 RS centrifuge, Heraeus) at 16 000 g at 4 °C. The biomass was stored at -20 °C.

Preparation of S-layer glycoprotein. S-layer glycoprotein of *B. stearothermophilus* NRS 2004/3a was prepared according to the standard protocol (Messner & Sleytr, 1988b).

Separation experiments. S-layer glycoprotein from *B. stearothermophilus* NRS 2004/3a was solubilized at a concentration of 30 mg ml⁻¹ in 0.1 M NaCl, applied to a Sephacryl S-1000 SF (Pharmacia) column (1 × 120 cm) and eluted with 0.1 M NaCl. Fractions of 1 ml were collected at a flow rate of 0.3 ml min⁻¹ and the elution profile was recorded simultaneously for carbohydrates, by a refractive index detector, and for protein, by a UV monitor at 280 nm. Appropriate pools were assayed for quantitative carbohydrate and amino acid composition by HPAEC-PED and amino acid analysis on a Biotronik LC3000

amino acid analyser, respectively. The pool containing mainly GlcNAc, Glc and ManpA2,3(NAc)₂ was rechromatographed under identical conditions in order to remove residual coeluting S-layer glycoprotein.

In another approach, separation between the ManpA2,3(NAc)₂-containing glycan and the S-layer glycoprotein of *B. stearothermophilus* NRS 2004/3a was achieved on an RP-4 column (Nucleosil 300-7 RP4 140813b, 4 × 125 mm; Machery & Nagel), equilibrated with 0.1% (v/v) TFA in MilliQ water. For separation of the two glycan components, a linear gradient of acetonitrile was applied (0–100% in 95 min) at a flow rate of 1 ml min⁻¹. The A₂₂₀ of the eluate was monitored and the eluate was collected in 1 ml aliquots.

Preparation of cell walls. Cells from the mid-exponential phase (wet wt, 100 g) obtained by continuous cultivation of *B. stearothermophilus* NRS 2004/3a were suspended in 250 ml cold distilled water and broken by ultrasonication using four cycles of 4 min, each at 4 °C. The homogenate was centrifuged at 48 400 g for 25 min at 4 °C. The supernatant was discarded and sedimenting broken cells were removed by gentle shaking with 1 M NaCl. The remaining pellet containing whole cells was resuspended in 150 ml cold water and repeatedly sonicated for 4 min. Combined fractions of crude cell walls from the ultrasonication steps were collected and diluted to a total volume of 250 ml with 1 M NaCl and centrifuged at 3020 g for 10 min. Remaining debris was removed and fractions sedimenting between 3020 and 48 400 g were collected, suspended in 200 ml distilled water and heated at 100 °C for 20 min. The suspension of crude cell walls was stored frozen at -20 °C.

Degradation of protein. After thawing, the cell-wall suspension was mixed with 250 ml 2 M sodium acetate buffer, pH 5.9, at room temperature, incubated with 20 mg ribonuclease at 37 °C for 3 h in a rotary shaker (150 r.p.m.), and then centrifuged for 1 h at 17 700 g (Araki *et al.*, 1972). The pellet was resuspended in 200 ml pre-warmed 50 mM sodium phosphate buffer, pH 7.5, and then digested with 20 mg trypsin for 2 h at 37 °C. The precipitate collected from the trypsin digest by centrifugation at 10 000 g for 1 h was treated with 250 ml 0.4% SDS at room temperature for 1 h, washed six times with distilled water (48 400 g, 20 min) and lyophilized, giving approximately 1 g of cell walls.

Re-N-acetylation. Since partial de-N-acetylation of N-acetyl amino groups might have occurred during the previous preparation steps, re-N-acetylation of cell walls of *B. stearothermophilus* NRS 2004/3a was carried out by shaking 1 g of the sample in a solution containing 8.4 g NaHCO₃, 5 ml acetic acid anhydride and 100 ml distilled water at 4 °C, overnight. Then the insoluble material was collected by centrifugation (48 400 g, 20 min), washed twice with distilled water, and lyophilized.

Lysozyme digestion. Lysozyme sensitivity of *B. stearothermophilus* NRS 2004/3a was examined by addition of 20 µl enzyme (1 mg ml⁻¹) to 2 ml of a suspension of cells from the mid-exponential phase in 50 mM Tris/HCl buffer, pH 7.2. The degradation of peptidoglycan was measured by the decrease of OD₆₀₀ of the sample over a time course of 90 min. Re-N-acetylated cell walls (approx. 1.15 g) were degraded at 37 °C for 48 h in a mixture containing 130 mg lysozyme (Sigma), 50 mM Tris/HCl buffer, pH 7.2 and 1% (w/v) sodium azide in a final volume of 400 ml (Amano *et al.*, 1977). The lysozyme digest was concentrated to 50 ml by evaporation at 30 °C, and dialysed (size cut-off of dialysis tubing, 12–16 kDa) against 5 l distilled water. The dialysis was repeated

five times with a total duration of 36 h. After separation of precipitating material from the non-dialysable fraction by centrifugation, the supernatant was lyophilized giving the crude peptidoglycan preparation.

Isolation of the secondary cell-wall polymer from the peptidoglycan preparation. The lyophilized soluble fraction from the lysozyme digest was dissolved in 5 ml 50 mM NH_4HCO_3 solution and applied to an equilibrated Sephadex G-50 column (2.6×100 cm). The column was eluted with 50 mM NH_4HCO_3 at a flow rate of 2 ml min^{-1} , and the elution pattern was monitored by measurement of refraction index and A_{280} . Fractions of 5 ml were collected and the total elution volume was 605 ml. These fractions were assayed for rhamnose, *N*-acetylglucosamine and amino acids (Christian *et al.*, 1993; Bock *et al.*, 1994; Altman *et al.*, 1996; Schäffer *et al.*, 1996). Those giving a positive colour reaction were pooled, concentrated on a vacuum evaporator at 30°C , dialysed five times against 3 l distilled water, and lyophilized.

The polysaccharide fraction obtained by chromatography on Sephadex G-50 was further purified by reversed-phase HPLC (RP-HPLC) on a semipreparative RP-18 column (Nucleosil 120-3C18, 8×125 mm; Machery & Nagel) equipped with an RP-18 guard column (Nucleosil 120-3C18, 8×40 mm; Machery & Nagel). The secondary cell-wall polysaccharide was separated from peptidoglycan and other contaminating proteins by using a $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ gradient, containing 0.1% TFA (0–15% CH_3CN for 0–45 min; 15–100% CH_3CN for 45–47 min; 100% CH_3CN for 47–50 min; 100–0% CH_3CN for 50–55 min; 0% CH_3CN for 55–60 min) at a flow rate of 3 ml min^{-1} . For some experiments, addition of TFA to the CH_3CN gradient was omitted. Absorbance was detected at 220 nm and fractions of 3 ml were collected. Fractions were combined according to the UV-elution profile and the qualitatively determined carbohydrate content. The pool containing the peptidoglycan-associated polysaccharide was finally lyophilized, yielding approximately 165 mg of secondary cell-wall polymer.

For the NMR experiments (see Table 1) the following preparations were used: preparation I (9.1 mg per ml D_2O) – secondary cell-wall polymer, purified by RP-HPLC in the presence of TFA in the CH_3CN gradient (retention interval 20–27 min); preparation II (21.0 mg per ml D_2O) – secondary cell-wall polymer, purified by RP-HPLC without addition of TFA to the gradient (retention interval 3–15 min); preparation III (5.0 mg per ml D_2O) – during purification of preparation II the material eluting from the column at 47–49 min was rechromatographed on the same RP-HPLC system using a modified CH_3CN gradient of 0–100% in 95 min. The material eluting at 11–18 min was collected and lyophilized. By amino acid analysis and HPAEC-PED it was demonstrated that it represented the cleaved pure peptidoglycan portion of that complex.

NMR experiments. The ^{13}C NMR spectra (100.62 MHz, attached proton test) were recorded on a BRUKER Avance DRX 400 WB NMR spectrometer equipped with a ^1H , ^{13}C observe dual probe. All other spectra were recorded with a BRUKER Avance DRX 600 NMR spectrometer using either a 5 mm broadband inverse probe with *z*-gradients or a 5 mm triple probe (^1H , ^{13}C , broadband) with *x*, *y*, *z*-gradients at frequencies for ^1H at 600.13 MHz, for ^{13}C at 150.90 MHz, and for ^{31}P at 242.94 MHz, respectively. A series of the following experiments were performed for the samples in D_2O solution.

Double quantum filtered correlation spectroscopy (DQF-COSY) with gradient coherence selection according to the scheme of Davis *et al.* (1991) [2048 data points in f_2 , 512 data

points in f_1 , sweep width 3000 Hz, 1 ms sine-shaped gradient pulses with 20% maximum amplitude, absorption mode in f_1 using time proportional phase incrementation (TPPI)]; total correlated spectroscopy (TOCSY) with 3-9-19 WATERGATE (Piotto *et al.*, 1992; Sklenar *et al.*, 1993) solvent suppression (2048 data points in f_2 , 512 data points in f_1 , sweep width 4500 Hz, 120 ms MLEV17 spin lock, 1 ms sine-shaped gradient pulses with 20% maximum amplitude, absorption mode in f_1 using TPPI); nuclear Overhauser effect spectroscopy (NOESY) with 3-9-19 WATERGATE (2048 data points in f_2 , 512 data points in f_1 , sweep width 4500 Hz, 150–500 ms mixing time, 1 ms sine-shaped gradient pulses with 20% maximum amplitude, absorption mode in f_1 using TPPI); sensitivity enhanced ^1H , ^{13}C -HSQC with echo/anti-echo selection (Kay *et al.*, 1992; Schleucher *et al.*, 1994) (2048 data points and 3000 Hz sweep width in f_2 , 256 data points and 15000 Hz sweep width in f_1 , 1 ms sine shaped gradient pulses with 80% maximum amplitude, globally optimized alternating-phase rectangular pulses (GARP) ^{13}C decoupling during acquisition, optimized on CH groups); gradient selected ^1H , ^{13}C -HMBC (hetero multiple bond correlation spectroscopy) (Hurd & John, 1991) with and without a low-pass *J*-filter (2048 data points and 3000 Hz sweep width in f_2 , 512 data points and 27150 Hz sweep width in f_1 , 1 ms sine-shaped gradient pulses with 50% maximum amplitude, long-range coupling 5 or 6 Hz).

In addition for the samples in 90% $\text{H}_2\text{O}/10\%$ D_2O the DQF-COSY and the experiments using the 3-9-19 WATERGATE solvent suppression scheme were recorded applying magic angle gradients (van Zijl *et al.*, 1995) with 1 ms sine-shaped gradients of equal amplitude along every axis and a maximum amplitude of 40% in one direction.

Experiments using ^{31}P frequency include direct observed phosphorus NMR spectra with proton decoupling, ^1H NMR spectra with ^{31}P GARP decoupling and two-dimensional gradient selected ^1H , ^{31}P -HMBC spectra (Hurd & John, 1991).

All the processing was done off line on Silicon Graphics workstations using the BRUKER software XWIN-NMR 2.0. All two-dimensional spectra were zero-filled in f_1 , doubling the data points and multiplied in both dimensions with a 90°-shifted square sine window function. The spectra were phase corrected in absorption mode in both dimensions except the HMBC spectra, which were calculated to magnitude mode. In addition to the BRUKER software, for the analysis the programs PRONTO 970306 (provided by M. Kjaer, F. M. Poulsen and the Carlsberg Research Center, Copenhagen, Denmark) and AZARA 2.0 (provided by W. Boucher and the Department of Biochemistry, University of Cambridge, UK) were used also on Silicon Graphics workstations.

RESULTS

Preparation of the S-layer glycoprotein

The S-layer glycoprotein of *B. stearothermophilus* NRS 2004/3a was prepared by the standard purification procedure (Messner & Sleytr, 1988b) including removal of the cytoplasmic membrane by treatment with Triton X-100 and extraction of the S-layer glycoprotein using 5 M guanidine hydrochloride. Carbohydrate composition of that material, as determined by HPAEC-PED, revealed a total rhamnose content of 185 nmol (mg S-layer glycoprotein) $^{-1}$ (~4.5%, w/w), originating from the rhamnan S-layer glycan, and 8 nmol each of GlcNAc and Glc per mg, indicating the presence of trace amounts

(~0.5%, w/w) of ManpA2,3(NAc)₂-containing glycan (Messner *et al.*, 1987).

Separation of the ManpA2,3(NAc)₂-containing glycan from S-layer glycoprotein

To prove that the ManpA2,3(NAc)₂-containing glycan chains are not covalently linked to the S-layer glycoprotein of *B. stearothermophilus* NRS 2004/3a, separation experiments using improved chromatographic techniques were performed. S-layer glycoprotein prepared by the standard purification procedure was subjected to gel filtration on a Sephacryl S-1000 SF column; this resulted, after rechromatography of the appropriate pool, in a complete separation of the intact high-molecular-mass S-layer glycoprotein with the glycosidically bound rhamnan, and a peak of lower apparent molecular mass representing the ManpA2,3(NAc)₂-containing material. Amino acid analysis of the latter pool revealed GlcNAc and a substance which showed the retention time of histidine as constituents. In the previous study (Messner *et al.*, 1987) this material had been identified as ManpA2,3(NAc)₂. Additionally, the presence of the constituents GlcNAc and Glc and of trace amounts of ManNAc was confirmed by HPAEC-PED.

Separation of the ManpA2,3(NAc)₂-containing material from the S-layer glycoprotein was also performed by RP-HPLC on an RP4 column with a CH₃CN-TFA gradient. The ManpA2,3(NAc)₂-containing glycan eluted at a CH₃CN concentration of approximately 31% (v/v), whereas the S-layer glycoprotein was collected in the gradient at 45% CH₃CN, demonstrating that there is no covalent linkage of this glycan-containing material to the S-layer glycoprotein. This result supports the observation from the gel-permeation chromatography experiment.

Isolation of the ManpA2,3(NAc)₂-containing glycan from peptidoglycan

To collect sufficient amounts of ManpA2,3(NAc)₂-containing glycan for analysis of its linkage region we changed the purification strategy for this polysaccharide fraction. The currently used modified procedure essentially follows the method of Araki *et al.* (1972). After isolation of the cell walls, to which the ManpA2,3(NAc)₂-containing glycan is still attached, the preparation was incubated for 48 h at 37 °C with lysozyme (Amano *et al.*, 1977) to break down the peptidoglycan sacculus completely. The reaction mixture was subjected to gel filtration on a Sephadex G-50 column and appropriate polysaccharide-containing fractions were collected. Further purification was performed by HPLC using a CH₃CN gradient, containing 0.1% (v/v) TFA for improved separation. Omission of TFA in the HPLC gradient gave poorer resolution but higher recovery of the intact cell-wall polymer-peptidoglycan complex (Fig. 1). Appropriate fractions were collected and examined by HPAEC-PED and amino acid analysis. Quantification showed that

20–25% of the MurNAc residues were randomly substituted with the ManpA2,3(NAc)₂-containing polysaccharide. These fractions were subjected to NMR analysis (for details see Methods).

NMR characterization of the secondary cell-wall polymer

The glycan structure was derived from one- and two-dimensional NMR experiments recorded on a 400 and a 600 MHz spectrometer. The structure of the repeating unit was confirmed to consist of four sugars in the described arrangement (Messner *et al.*, 1987). Since chemical shift values and line width of the observed NMR signals showed a strong pH dependence, the reinvestigation of the glycan was performed at two different pH values. At low pH values (~pH 2.0) the proton signals were sharp and the anomeric region was well resolved. At higher pH values (~pH 3.5), however, the signal half width increased and Glc_p and Glc_pNAc anomeric protons of the polysaccharide chain overlapped. In the ¹³C NMR spectra, particularly the C-5 and C-6 signals of the ManpA2,3(NAc)₂ units were shifted. In addition to the reinvestigated and completed ¹³C chemical shift data, an assignment of the ¹H spin systems was possible by gradient-enhanced (ge) homo- and heteronuclear two-dimensional NMR experiments (Table 1). The glycosidic linkages were confirmed from ge-HMBC experiments by means of three-bond correlation of the anomeric protons to the glycosidic bond carbons and vice versa, thus providing a non-empirical verification of the reported sequence.

Starting from the proton chemical shift data (Table 1, Fig. 2) of the anomeric proton from sugar A an additional cross-peak at lower intensity could be identified in the DQF-COSY experiment. Eventually it led to a complete set of ¹H and ¹³C signals belonging to a ManpA2,3(NAc)₂ residue which showed significant chemical shift differences for the ¹H signals in positions 2 to 5 and especially the ¹³C signal of C-4. The observed upfield shift at the C-4 ¹³C shift value, as well as the H-4 of that ManpA2,3(NAc)₂ unit A', confirmed this unit as the terminal sugar at the non-reducing end of the polysaccharide chain. Integration of the well-resolved cross-peaks in the HSQC experiment (Fig. 3) for this terminal sugar and comparison with the cross-peak volume of the corresponding signals in the polysaccharide chain showed a ratio of approximately 1:5, indicating a chain length of approximately six repeating units.

To identify the linkage region between the repeating units and the peptidoglycan present in the sample, proton spectra were recorded with simultaneous ³¹P decoupling to detect signals of phosphorylated sugars. In the anomeric region at approximately 5.43 p.p.m. a collapse of a doublet splitting (³J_{H,P} ~ 6 Hz) could be seen in preparation I (see Methods for the different preparations). In preparation II a significant reduction of the line width for the signal at the same chemical shift was observed. A direct ³¹P spectrum with proton

Fig. 1. Final purification of the secondary cell-wall polymer from *B. stearothermophilus* NRS 2004/3a by RP-HPLC on a semipreparative RP-18 column using a $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ gradient without TFA.

decoupling gave two signals, at 1.33 and -0.69 p.p.m., respectively, in agreement for a diphosphate bridge.

Analysis of the whole spin system starting from this phosphorylated anomeric signal at 5.43 p.p.m. showed the unit designated D' to be an αGlcNAc residue which, based on the carbon chemical shifts, was substituted in position 3. Due to the low intensity of the signals for this unit, further investigation was a very tedious task. To resolve the spectral overlap, experiments were performed in 90% $\text{H}_2\text{O}/10\%$ D_2O solution (Fig. 4). As the whole system contained a number of amino sugars, additional information could be gained by NMR experiments in H_2O from the slowly exchanging NH signals in a chemical shift region far from the carbohydrate signals. In a NOESY spectrum with suppression of water signal by a 3.9-19 WATERGATE sequence, a cross peak could be identified starting from the NH signal of the phosphorylated GlcNAc to an anomeric proton of another sugar, again implying a $1 \rightarrow 3$ linkage. This new sugar was an amino sugar with an NHAc group in position 2. Based on the recorded anomeric one-bond CH coupling constant of 163 Hz and a small <2 Hz $^3J_{\text{H,H}}$ coupling constant of this sugar, a *manno* system carrying at least one NHAc group in position 2 can be supposed. Since the whole spin system could not be identified, no linkage information to the polysaccharide chain is available. However, since the chemical shift values observed for this unit designated C' are almost indistinguishable from that of unit C, it is fair to assume that units C' and D' belong to a repeating unit linked via pyrophosphate, thus indicating a blockwise attachment of the polysaccharide chain.

Continuing the NMR analysis from the phosphorylated anomeric signal towards the reducing end, a two-dimensional $^1\text{H}, ^{31}\text{P}$ HMBC spectrum was recorded showing signals at two different phosphorus frequencies. In addition to the anomeric proton and H-2 from the αGlcNAc unit D' at $\delta(^{31}\text{P}) = 1.33$ p.p.m. a signal set for two protons characteristic for a CH_2 group was detected [$\delta(^1\text{H}) = 4.073$ and 3.994 p.p.m., $\delta(^{31}\text{P}) =$

-0.69 p.p.m.], implying a linkage to a C-6, most probably of a muramic acid residue. Due to limitations in sensitivity (broad signals and additional ^1H , ^{31}P couplings) no direct connection from this H-6 to H-5 or C-5 of $\alpha/\beta\text{MurNAc}$ could be established. However, considering the different shifts of protons 4, 5 and 6 of $\alpha/\beta\text{MurNAc}$ in the intact polysaccharide-peptidoglycan preparation (Table 1, preparation II) to those shifts of the peptidoglycan alone (preparation III) indicates MurNAc to be phosphorylated in position 6 in the intact cell-wall polymer.

The signals belonging to the peptidoglycan moiety were visible in all spectra and were identified by comparison to spectra from the separated peptidoglycan portion alone. A detailed structural analysis of this part of the sample by NMR analysis was impossible due to the inherent heterogeneity of the sample. In about 60% of the intact cell-wall polymer (preparation II), the oligosaccharide-carrying glycan strands of the peptidoglycan consisted of disaccharide $\beta\text{-GlcNAc-(1} \rightarrow 4)\text{-}\alpha/\beta\text{-MurNAc}$ units. The rest contained larger fragments of the peptidoglycan saccharide strands (multiples of the disaccharide units in a random order; see Fig. 2). No attempts were made to assign every single signal from the peptidoglycan portion. A partial assignment of the peptidoglycan moiety was facilitated by comparison to reported values of synthetic models (Kantoci *et al.*, 1989). Alanine, glutamine and diaminopimelic acid were again assigned using ge-TOCSY, ge-DQF-COSY and ge-HSQC spectra. The peptide sequence of the sample was compatible with that of the *Bacillus* peptidoglycan chemotype A1 γ (Schleifer & Kandler, 1972; Archibald, 1989).

DISCUSSION

The walls of Gram-positive bacteria usually contain peptidoglycan and different accessory polymers such as teichoic acids, teichuronic acids, neutral or charged polysaccharides, proteins (e.g. S-layer proteins), and in some cases even covalently bound lipids (for review see Archibald, 1989). Teichoic acid and teichuronic acid are covalently linked to the peptidoglycan throughout the thickness of the wall (Ward, 1981). Teichoic acids contain charged phosphate groups and, often, charged amino acid groups whereas teichuronic acids contain charged carboxyl groups. Although they are often described as accessory or secondary wall polymers, their presence is required for the ordered assembly of wall material and for normal wall function. Due to their functional groups they contribute to the overall surface charge of the cells and also to specific surface properties (for reviews see Ward, 1981; Hancock, 1991).

For several *Bacillus* strains, secondary cell-wall polymers which contain cores with the structure $[\rightarrow 3)\text{-}\beta\text{-D-ManpNAc-(1} \rightarrow 4)\text{-}\beta\text{-D-GlcpNAc-(1} \rightarrow]$, connecting the oligosaccharide chain to the pyrophosphate group, have been described (Kaya *et al.*, 1984; Kojima *et al.*, 1985, 1988; Araki & Ito, 1989). Similar cores were also found in typical ribitol and glycerol teichoic acids of

Table 1. ^1H and ^{13}C NMR chemical shift data (in p.p.m.) of the diacetamidodideoxyuronic-acid-containing glycan chain and the linkage region of *B. stearothermophilus*

For each preparation (I, II, III), the first row shows chemical shift data for ^1H relative to external acetone ($\delta = 2.225$ p.p.m.) and the second row shows chemical shift data for ^{13}C relative to external dioxane ($\delta = 67.40$ p.p.m.). All data were acquired at 300 K. Numbers in parentheses represent $^1J_{\text{H,C}}$ coupling constants in Hz. I, preparation I from HPLC separation with 0.1% (v/v) TFA in the CH_3CN elution gradient (6.4 mg in 0.7 ml D_2O , pH = 2.11); II, preparation II from HPLC separation without TFA (14.7 mg in 0.7 ml D_2O , pH = 3.32); III, preparation III containing only the peptidoglycan portion (3.5 mg in 0.7 ml D_2O , pH = 4.10).

	Carbohydrate signals						2-N-Acetyl			3-N-Acetyl		
	1	2	3	4	5	6	CO	CH_3	NH	CO*	CH_3^*	NH
A' β -ManpA2,3(NAc) $_2$ -(1 $\rightarrow$ I	4.932	4.509	4.104	3.725	4.070							
II	100.68	51.54	54.23	66.79	77.44	173.54						
A $\rightarrow$ 4)- β -ManpA2,3(NAc) $_2$ -(1 $\rightarrow$ I	4.909	4.482	4.070	3.665	3.909							
II	100.59	51.64	54.25	67.20	78.97		175.78		8.113†			8.254†
B $\rightarrow$ 6)- α -GlcP-(1 $\rightarrow$ I	4.929	4.403	4.321	3.969	4.112			2.066			1.921	
II	100.68	51.86	54.00	71.29	77.22	173.93	176.06	22.85		175.06	22.78	
II	4.900	4.360	4.295	3.930	3.970			2.057	8.263†		1.921	8.440†
II	100.59 (162.2)	52.12	54.15	71.50	78.97		175.97	22.84		174.91	22.76	
C $\rightarrow$ 4)- β -ManpA2,3(NAc) $_2$ -(1 $\rightarrow$ I	5.078	3.369	3.557	3.377	3.640	4.005/3.844						
II	99.86	71.92	73.26	69.57	71.92	69.06						
II	5.075	3.340	3.577	3.390	3.690	3.990/3.875						
II	99.44 (171.1)	72.00	73.22	69.41	71.72	68.70						
D $\rightarrow$ 3)- α -GlcPNAc-(1 $\rightarrow$ I	4.992	4.313	4.327	3.888	4.107			2.033			1.871	
II	100.49	52.34	54.00	72.32	77.22	173.08	175.58	22.70		174.60	22.64	
II	4.955	4.273	4.310	3.873	3.987			2.027	8.210†		1.864	8.440†
II	100.67 (163.6)	52.47	54.15	71.90	78.87		175.49	22.69		174.39	22.61	
E' $\rightarrow$ 4)- β -ManpA2,3(NAc) $_2$ -(1 $\rightarrow$ I	5.032	4.301							8.191†			
II	100.63 (163.0)	52.40										
D $\rightarrow$ 3)- α -GlcPNAc-(1 $\rightarrow$ I	5.109	3.926	3.801	3.596	3.596	3.813/3.738		1.973				
II	97.81	52.72	81.69	68.68	72.88	60.60	174.72	22.78				
II	5.087	3.895	3.819	3.565	3.690	3.804/3.745		1.971	8.340†			
II	97.59 (173.3)	52.72	81.84	68.92	72.48	60.58	174.66	22.76				
D' $\rightarrow$ 3)- α -GlcPNAc-(1 $\rightarrow$ P-O-P $\rightarrow$ ‡, §	5.429	4.036	3.930	3.605	3.845	3.842/3.766						
II	94.92	53.15	81.70	72.80	73.80	61.30						
II	5.431	4.032	3.893	3.590	3.847	3.840/3.746			8.294†			
II	94.91 (171.9)	53.17	81.81	72.30	73.80	61.30						
E β -GlcPNAc	4.538	3.740	3.560	3.406	3.419	3.937/3.752		7.720†				
II	101.39	56.75	74.50	71.19	76.97	61.90						
III	4.533	3.747	3.550	3.414	3.416	3.941/3.758		2.034	7.752			
III	101.18	56.82	74.24	71.01	76.90	61.91		23.00				
	Carbohydrate signals						2-N-Acetyl			3-O-I-Acetyl		
	1	2	3	4	5	6	CO	CH_3	NH	CH	CH_3	CO
F α -MurNAc												
II	5.227	3.824	3.737	3.541	3.855	4.165/4.090			7.912†	4.554	1.383	
III	91.08 (171.9)	54.45	77.10	74.50	71.82	64.30				78.39	18.69	
III	5.238	3.832	3.748	3.553	3.863	3.941/3.758		1.965	7.946	4.561	1.389	
III	90.99	54.47	76.95	74.24	71.86	61.08		23.07		78.27	18.62	176.11
G β -MurNAc												
II	4.632	3.722	3.859	3.466	3.583				8.373†	4.554	1.383	
III	95.12	56.46	76.30	75.73	74.50					78.39	18.69	
III	4.640	3.721	3.855	3.466	3.586					4.561	1.389	
III	95.82	56.77	76.15	75.43	74.35				8.355	78.27	18.62	176.11
	Peptide signals											
	CO	CH_α	NH	CH_β	CH_γ	CH_δ	CH_ϵ	NH ϵ	CO ω			
Ala												
II		4.327	8.378†	1.430								
III	175.43	50.51		17.53								
III		4.314	8.467	1.438								
III		50.57		17.57								
Ala												
II		4.277	8.633†	1.394								
III	176.16	50.83		16.99								
III		4.347	8.389	1.381								
III		50.23		17.38								
Glu§												
II			8.480†									
II			8.335†									
II		4.395	8.268†		2.180/1.980	2.370						
III		53.90			28.02	32.45						175.71
III		4.363	8.528		2.153/1.987	2.403						
III		53.69			28.02	32.40						
meso-diaminopimelic acid¶												
II										8.053†		
II										8.216†		
II										8.172†		
III	177.82	4.214			1.864/1.740	1.480/1.390	1.864/1.740	4.182				
III		54.29			31.01	22.34	31.01	55.12				
III		4.264	8.208		1.850/1.740	1.464/1.391	1.850/1.740	4.225		8.088		175.76
III		54.20			31.30	22.35	31.30	54.90				

* Assignments interchangeable.

† Preparation II in 0.7 ml 90% (v/v) H_2O /10% (v/v) D_2O .‡ δ (^{21}P) = 1.33 p.p.m. and -0.69 p.p.m. (relative to external 80% H_3PO_4 , $\delta = 0$ p.p.m.).§ Connected to a CH_2 group [δ (^1H) = 4.073, 3.994 p.p.m.] in preparation I.|| Preparation III in 0.7 ml 90% (v/v) H_2O /10% (v/v) D_2O .¶ δ values approximately, belonging to more than one residue.

Fig. 2. Proposed structure of the secondary cell-wall polymer–peptidoglycan complex. The capital letters indicate the appropriate units according to the assignment from the NMR experiments.

Fig. 3. ^1H , ^{13}C -ge-HSQC NMR spectrum of preparation II, core region and (inset) anomeric region.

Gram-positive organisms (for reviews see Munson & Glaser, 1981; Archibald, 1989). From the combined data of the structural and chemical characterization of the secondary cell-wall polymer of strain *B. stearotheophilus* NRS 2004/3a it can be concluded that the ManpA_{2,3}(NAc)₂-containing saccharide chains, which on average are composed of approximately six identical repeating units (Messner *et al.*, 1987), are linked via

pyrophosphate bridges to about 20–25 % of the muramic acid residues of the peptidoglycan sacculus (Fig. 2). Although during the chemical characterization of the secondary cell-wall polymer of *B. stearrowthermophilus* NRS 2004/3a, GlcNAc and trace amounts of ManNAc have been determined, the NMR data do not support the presence of a ManNAc → GlcNAc core disaccharide in this organism. On the other hand, its existence cannot

Fig. 4. TOCSY NMR spectrum (3-9-19 WATERGATE solvent suppression) of preparation II in 90% H₂O/10% D₂O, amide region.

be ruled out completely because the first two components of the repeating unit are GlcNAc and a manno-sugar system which would be similar to the signals for the postulated core in the NMR experiments (see Table 1). The existence of a comparable core disaccharide consisting of GlcNAc and ManpA2,3(NAc)₂ residues has been suggested in a preliminary report on an acidic cell-wall polysaccharide of *Propionibacterium acnes* (Nagaoka *et al.*, 1985), however, without further substantiation.

The identified linkage region of the ManpA2,3(NAc)₂-containing glycan, together with the observation that this glycoconjugate can be separated from the S-layer glycoprotein by improved chromatographic techniques, clearly demonstrates that this glycoconjugate is not covalently linked to the S-layer glycoprotein. It shows that there is only low, if any, specific affinity of the secondary cell-wall polymer to the intact S-layer protein. This observation is supported by the previous separation experiments of proteolytically degraded S-layer glycoprotein preparations, which resulted in a complete splitting of both carbohydrate structures (Messner *et al.*, 1987). Thus, our previous assumption of a possible glycoprotein nature of the ManpA2,3(NAc)₂-containing glycoconjugate should be changed.

In conclusion, this glycoconjugate represents a secondary cell-wall polymer of *B. stearothermophilus* NRS 2004/3a; this has also been observed with other *B. stearothermophilus* strains (Ries *et al.*, 1997; Egelseer *et al.*, 1998). Possible biological functions of this type of cell-wall constituent in bacilli have been recently discussed in the context of mediating binding of the S-layer to the peptidoglycan sacculus (Egelseer *et al.*, 1998; Sára *et al.*, 1998). Comparable functional analyses have not

been performed with the secondary cell-wall polymer of *B. stearothermophilus* NRS 2004/3a.

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