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A pyrophosphate bridge links the pyruvate-containing secondary cell wall polymer of *Paenibacillus alvei* CCM 2051 to muramic acid

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The peptidoglycan, the secondary cell wall polymer (SCWP), and the surface layer (S-layer) glycoprotein are the major glycosylated cell wall components of *Paenibacillus alvei* CCM 2051. In this report, the complete structure of the SCWP, its linkage to the peptidoglycan layer, and its physicochemical properties have been investigated. From the combined evidence of chemical and structural analyses together with one- and two-dimensional nuclear magnetic resonance spectroscopy, the following structure of the SCWP-peptidoglycan complex is proposed:
[(Pyr4,6)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→3)]$_{n\sim11}$-(Pyr4,6)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*NAc-(1→O)-PO$_2$-O-PO$_2$-(O→6)-MurNAc-

Each disaccharide unit is substituted by 4,6-linked pyruvic acid residues. Under mild acidic conditions, up to 50% of them are lost, leaving non-substituted ManNAc residues. The anionic glycan chains constituting the SCWP are randomly linked via pyrophosphate groups to C-6 of muramic acid residues of the peptidoglycan layer. ^{31}P NMR reveals two signals that, as a consequence of micelle formation, experience different line broadening. Therefore, their integral ratio deviates significantly from 1:1. By treatment with ethylenediaminetetraacetic acid, sodium dodecyl sulfate, and sonication immediately prior to NMR measurement, this ratio approaches unity. The reversibility of this behavior corroborates the presence of a pyrophosphate linker in this SCWP-peptidoglycan complex.

In addition to the determination of the structure and linkage of the SCWP, a possible scenario for its biological function is discussed.

***Keywords:* *Paenibacillus alvei*, secondary cell wall polymer (SCWP), surface layer (S-layer), peptidoglycan, NMR spectroscopy, structure determination**

***Abbreviations:* EDTA, ethylenediaminetetraacetic acid; HPAEC/PED, high-performance anion exchange chromatography with pulsed electrochemical detection; HSQC, heteronuclear single quantum correlation spectroscopy; NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; 2QF-COSY, double quantum-filtered correlated spectroscopy; RP-HPLC, reversed-phase high-performance liquid chromatography; SCWP, secondary cell wall polymer; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; S-layer, bacterial cell surface layer; TPPI, time proportional phase incrementation.**

Introduction

During the last decade, the application potential of crystalline bacterial cell surface layers (S-layers) in the fields of biotechnology, biomimetics and molecular nanotechnology has been extensively explored (for review see [1,2]). One of the areas investigated concerned the utilization of S-layer (glyco)-proteins as immobilization matrix for antigens, haptens and immunostimulatory substances, aiming at the development of model conjugate vaccines (for review see [3–5]). Among the bacterial strains used for immobilization studies was the mesophilic organism *Paenibacillus alvei* strain CCM 2051. *P. alvei* CCM 2051 is covered with an oblique S-layer lattice [6] consisting of identical glycoprotein subunits [7,8]. The

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glycan chains have been demonstrated to consist on average of twenty branched trisaccharide repeating units with the structure → 3)-β-D-Gal*p*-(1 → 4)[α-D-Glc-(1 → 6)]-β-D-Man-*p*NAc-(1 → . They are linked via the core heterosaccharide → 3)[GroA-(2 → O)-PO_2-(O → 4)-β-D-Man*p*NAc-(1 → 4)]-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 → 3)-α-L-Rha*p*-(1 →3)-β-D-Gal*p* in *O*-glycosidic linkage to tyrosine residues of the S-layer polypeptide [7,9].

Many *Bacillus* strains possess, in addition to glycosylated S-layer proteins, other glycoconjugates such as peptidoglycan-associated teichoic or teichuronic acids and secondary cell wall polymers (SCWP), exhibiting common linkage units (for reviews see [10–12]). One type of linkage unit is the disaccharide β-D-Man*p*NAc-(1 → 4)-β-D-Glc*p*NAc, which is bound via a phosphate group to muramic acid residues of the peptidoglycan layer [13]. In a few organisms, this disaccharide motif is extended to form longer chains with the alternating repeating unit → 3)-β-D-Man*p*NAc-(1 → 4)-β-D-Glc*p*NAc-(1 →. Frequently, the ManNAc residues of the glycan chain are substituted with constituents such as ribofuranose, as in the case of the SCWP of *Thermoanaerobacterium thermosaccharolyticum* strains ([14]; Schäffer, Kählig, Messner, unpublished observation), or pyruvic acid, occurring in the acidic polysaccharide of *Bacillus polymyxa* AHU 1385 [15] and in the SCWP of *Bacillus sphaericus* CCM 2177 [16].

Recently, immunological studies concerning cross-reactivity of S-layer preparations from different bacilli have shown that the S-layers of *Bacillus stearothermophilus* PV72/p2 [17] and *P. alvei* CCM 2051 do not immunologically cross-react [Schäffer, Sleytr, Messner, unpublished observation], making them promising candidates for immunization protocols using a combination of different S-layer matrices. These S-layer preparations usually contain strain-specifically varying amounts of non-covalently attached SCWP [18–21]. Thus, in order to fully understand the immune response to the S-layer matrix and, beyond that, to the S-layer-based conjugate, not only the S-layer preparation itself, but also these peptidoglycan-associated compounds have to be characterized prior to the construction of a model vaccine. Therefore, we have focussed our efforts on the structure elucidation of the SCWP of *P. alvei* CCM 2051, including its linkage to muramic acid residues of the peptidoglycan layer, and its physicochemical properties.

Materials and methods

Analytical methods

Carbohydrate analysis by HPAEC/PED was performed after hydrolysis of samples for 4 h at 110°C with 2.46 M trifluoroacetic acid as described recently [22]. For qualitative detection of carbohydrates, the thymol reagent was used, with amino sugars typically giving a yellow colour reaction [23]. Amino acid analysis with concomitant identification of muramic acid was done after hydrolysis of samples for 4 h at 110°C with 6 M HCl, containing 0.2% thioglycolic acid, on a Biotronic LC3000 amino acid analyzer [22]. The phosphate content was determined colorimetrically [24].

Growth of the bacteria

Paenibacillus alvei CCM 2051 was obtained from the Czech Type Culture Collection (Brno, Czech Republic) and was grown at 32°C in continuous culture on a complex standard medium as recommended by the Type Culture Collection, containing 0.8% (w/v) Difco® Nutrient Broth, 0.25% (w/v) yeast extract, and 0.25% (w/v) glucose, in a 15-liter Biostat C® fermenter (Braun). The pH of the culture was regulated at 7.0 ± 0.15 by addition of 2 M H_2SO_4 and the fermentation was carried out with an oxygen partial pressure of 50% (v/v) and an average dilution rate D of $0.15\ h^{-1}$. Cells were separated from culture broth by continuous centrifugation (Sepatech 17 RS contifuge, Heraeus) at 16 000 ×**g** at 4°C. The biomass was stored at −20°C.

Cell walls

Cells of *P. alvei* CCM 2051 collected from 40 l of continuous culture broth (wet weight, 75 g) were washed with distilled water in order to remove residual media components and subsequently suspended into 150 ml of distilled water. Breaking of cells was achieved by sonicating the suspension at 4°C in cycles of three, six and eight min, respectively. Cell walls were sedimented at 48 400 ×**g** and combined cell walls from the sonication steps were mixed with 200 ml of cold 1 M NaCl. The fraction sedimenting between 3200 ×**g** and 48 400 ×**g** was collected and the resulting crude cell walls were suspended into 150 ml of distilled water and heated at 100°C for 20 min. After cooling down the suspension on ice, it was treated with ribonuclease (15 mg; 3 h 37°C), trypsin (15 mg; 2 h 37°C) and 0.4% SDS (150 ml; 1 h room temperature) for degradation of protein. After six washes of the pellet it was resuspended in 25 ml of distilled water containing 2.1 g $NaHCO_3$, and 1.25 ml of acetic acid anhydride were added to ascertain full *N*-acetylation of amino groups. This treatment is a prerequisite for full lysozyme sensitivity [25]. Degradation of protein and re-*N*-acetylation are described in detail elsewhere [21]. The material was lyophilized and stored at 4°C until use.

Lysozyme digestion of re-*N*-acetylated cell walls

250 mg of cell walls of *P. alvei* CCM 2051 obtained after re-*N*-acetylation were digested with 35 mg of lysozyme (Sigma) in a final volume of 100 ml of 50 mM Tris/HCl buffer, pH 7.2, containing 1% NaN_3, for 48 h at 37°C under gentle stirring. The reaction mixture was concentrated on a vacuum evaporator at 30°C and dialyzed six times against three litres of distilled water, each, at 4°C. The non-degradable part of the mixture was sedimented at 48 400 ×**g** and the supernatant, giving the crude peptidoglycan preparation, was further investigated.

Isolation of the SCWP-peptidoglycan complex from the peptidoglycan preparation

The lyophilized, soluble fraction from the lysozyme digest was dissolved in 3 ml of 50 mM NH_4HCO_3 solution and applied to a Sephadex G-50 column (2.6 × 100 cm; Amersham Pharmacia Biotech; fractionation range: 1000–30 000). 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 simultaneous on-line measurement of the refractive index (RI) and the absorbance at 280 nm. Fractions of 5 ml were collected up to a total elution volume of 605 ml. Fractions were pooled according to their colour reaction with thymol and the RI and $A_{280\,nm}$ profiles. Pools were concentrated on a vacuum evaporator at 30°C, dialyzed until salt-free, and lyophilized.

The high-molecular-weight, polysaccharide–containing material obtained after chromatography on Sephadex G-50 was further fractionated at a flow rate of 0.3 ml min^{-1} on a Bio-Gel P-30 column (1.5 cm × 120 cm; Bio-Rad; fractionation range: 2500–40 000), with 0.1 mM NaCl as eluent. The eluate was monitored as described for the Sephadex G-50 chromatography. Carbohydrate-containing fractions of 2 ml, each, were pooled, lyophilized and finally purified by 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 SCWP-peptidoglycan complex was separated from peptidoglycan fragments and other residual protein contaminants in an CH_3CN/H_2O gradient at a flow rate of 3 ml min^{-1} [21]. Absorbance was detected at 220 nm and fractions of 3 ml were collected. Fractions were combined according to the UV-elution profile and their carbohydrate content. The pool containing the peptidoglycan-associated SCWP was finally lyophilized and used for the NMR experiments.

NMR experiments

A solution of the peptidoglycan-associated SCWP (approximately 20 mg ml^{-1}) in 99.95% D_2O, exhibiting a pH value of 3.0, was used. The NMR spectra were recorded at 323 K non-spinning in 5 mm tubes at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C on a Bruker Avance DRX 500 spectrometer using a 5 mm TXI probe with triple axis gradient coils. In order to compare with published work [16], spectra were referenced with internal trimethylsilyl propionate-d_4 at 0 ppm for proton and at −2.0 ppm for carbon atoms. Typical 90° hard pulse durations were 7 µs (^1H) and 17.5 µs (^{13}C); 90° pulses in decoupling sequences were set to 100 µs (^1H, WALTZ) and 80 µs (^{13}C, GARP). Repetition intervals were 2 s and gradient durations were 1.5 ms.

Double quantum filtered homonuclear 2D correlated ^1H spectra (2QF-COSY) were acquired using pulsed field gradients for coherence selection [26]. In NOESY, an 80 ms mixing interval with a 7 G cm^{-1} homospoil pulse was used. Pure phase detection was achieved by the TPPI method [27]. Proton detected heteronuclear correlated 2D-NMR spectra using pulsed field gradients (30 G cm^{-1} maximum strength) for coherence selection and the echo/anti-echo procedure [28] to obtain pure phase spectra (HSQC with sensitivity improvement [29] and with or without proton decoupling), were performed recording 1024(f1) × 2048(f2) complex data points.

Typically, the obtained data matrices were zero filled to 4096 × 4096 and, past Fourier transform in f2 and linear prediction data filling in f1, finally Fourier transformed in the f1 dimension [30]. For the linear prediction, 32 auto-regressive coefficients obtained with the covariance method were used [31].

^{31}P spectra were acquired with proton decoupling (WALTZ) in a 5 mm BBO probe with a single z-gradient coil at 202.46 MHz and referenced to external ortho-phosphoric acid at 0 ppm, ^{31}P 90° pulses were 10.5 µs. In selective $^1H\{^{31}P\}$ decoupling difference spectra the differences were calculated from alternating scans. The selective decoupler field strength ($B_1\gamma$) was approximately 25 Hz. 2D $^1H/^{31}P$ correlated spectra were recorded with proton detection using the Bruker pulse program *invieags* optimized for a heteronuclear coupling constant of 8 Hz and an average relaxation time of 50 ms. 10 mg ml^{-1} EDTA and 20 mg ml^{-1} SDS were added to the sample, which was then sonicated for 2 min immediately prior to ^{31}P NMR measurements [32].

Results

Isolation of the SCWP-peptidoglycan complex from *P. alvei* CCM 2051

A SCWP-peptidoglycan complex was isolated from a crude peptidoglycan preparation of *P. alvei* CCM 2051 by chromatographic methods. The material, eluting in the void volume of the Sephadex G-50 column, was spread into two major peaks on a Bio-Gel P-30 column. About 20% of the chromatographed material was recovered from the void volume (pool I). It had a carbohydrate content of less than 5%, but gave high absorbance at 280 nm, attributable to coeluting proteins and various peptidoglycan-derived fragments. From the elution behaviour of pool II, its molecular mass was estimated to be significantly less than 40 000. Pool II corresponded to 50% of the chromatographed material and gave a strongly positive colour reaction when analyzed with thymol reagent but had, in contrast to pool I, low A_{280}. Appropriate polysaccharide-containing fractions were collected and pool II was finally purified by RP18-HPLC. The material of interest eluted as a single, rather broad peak in a retention interval between 6 and 11 min, which corresponded to an CH_3CN concentration of 2–4%. The total yield of the purified SCWP-peptidoglycan complex was 17 mg, implicating that it constitutes a proportion of approximately 7% of the cell wall of *P. alvei* CCM 2051. Examination of this material by HPAEC-PED and amino acid analyses revealed the presence of ManNAc and

GlcNAc in the molar ratio of approximately 1:1.2, containing additional GlcNAc residues from the peptidoglycan matrix. The other common peptidoglycan constituents muramic acid, glutamic acid, alanine, and diaminopimelic acid, were found in the molar ratio of 0.9:1:1.5:1. This finding provided strong evidence that the isolated SCWP of *P. alvei* CCM 2051 was intact and thus still peptidoglycan-associated. For the calculation of the molar ratios of the individual constituents, diaminopimelic acid was arbitrarily set to a value of 1. This RP18 pool was subjected to structure elucidation by NMR spectroscopy.

NMR analysis of the SCWP-peptidoglycan complex

The structure of the polysaccharide repeating unit of the SCWP from *P. alvei* CCM 2051 was elucidated by one- and two-dimensional NMR techniques and found to be identical to that of *Bacillus polymyxa* AHU 1385 [15]. As in strain AHU 1385, the acidic polymer fraction additionally contained muramic acid. However, for that polymer the linkage between the saccharide and the muramic acid-containing peptidoglycan portion has not been determined. Based on the ^{13}C NMR data for the acidic polysaccharide of *B. polymyxa* AHU 1385 [15], the $^{1}H/^{13}C$ NMR data for both the backbone carbohydrate structure of the polymer preparation from *T. thermosaccharolyticum* E207-71 [14], and the peptidoglycan portion of the SCWP of *B. stearothermophilus* NRS 2004/3a [21], structure determination of the SCWP from *P. alvei* CCM 2051 was straightforwardly achieved by means of NMR spectroscopy. Proton and carbon (Figures 1A,B) shifts for the fully pyruvylated and depyruvylated polysaccharide are shown in Table 1. The chemical shifts for the muramic acid-containing portion concur with those reported previously for the SCWP of *B. stearothermophilus* NRS 2004/3a [21], and are thus not presented in Table 1. Prolonged exposure of the polymer sample to acidic conditions, as obtained upon solvatation of the SCWP-peptidoglycan complex in D_2O, resulted in a spectral change, indicating a loss of about 50% of pyruvate residues (Figure 1C) (for comparison see [14]).

The linkage between the saccharide and the peptidoglycan portion could not be determined directly. Two signals with an integral ratio of approximately 10:1 were found in the ^{31}P NMR spectrum (Figure 2). In $^{1}H/^{31}P$ correlated spectra, only the major ^{31}P signal ($\delta = -0.69$) showed a connectivity to the anomeric proton of an amino sugar at 5.43 ppm, while no connectivity of the minor one ($\delta = 1.33$) was detectable. Also from 1D $^{1}H\{^{31}P\}$ decoupling difference spectra it was evident that the only detectable scalar coupling of the major phosphorus signal was to proton 1 of the same amino sugar (Figure 3), thus excluding a teichuronic acid-like structure.

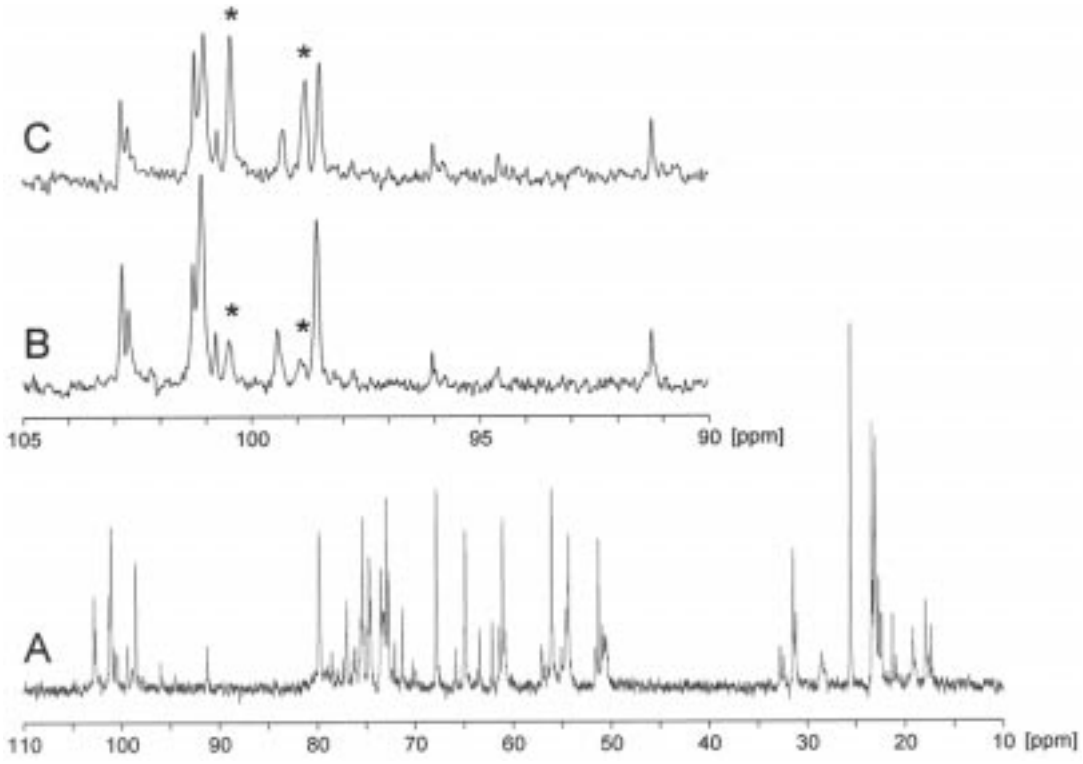

Figure 1. ^{13}C-NMR spectrum of the SCWP-peptidoglycan complex isolated from *P. alvei* CCM 2051. **A**, spectrum acquired upon approximately 10 h of recording time at 40°C; **B**, spectral extension of the anomeric carbon region of spectrum A; **C**, spectral change in the anomeric carbon region observed after approximately 100 h of recording time at 40°C. The signals corresponding to a decrease in pyruvylation of the repeating unit of the SCWP are marked with asterisks.

Table 1. Partial ^{13}C and ^{1}H chemical shift data (in ppm) of the SCWP of *P. alvei* CCM 2051, recorded at 323 K in D_2O[a]

	Glycosyl unit			
	→ 3)-4,6Pyr-β-D-ManNAc-[b,c]	*→ 4)-β-D-GlcNAc-*[d]	*→ 3)-β-D-ManNAc-*[e]	*→ 4)-β-D-GlcNAc-*[e]
Carbon/				
1	101.10 (163)[f]	98.57 (163)[f]	100.50	98.93
2	51.30	56.08	50.80	56.13
3	74.70	73.52	79.97	73.52
4	67.84	79.86	65.95	79.77
5	72.95	75.40	77.17	75.48
6	64.90	61.09	61.50	61.10
1′[g]	n.d.[h]			
2′[g]	102.83			
3′[g]	25.57			
Proton/				
1	4.85	4.59	4.81	4.59
2	4.70	3.73	4.67	3.75
3	4.19	3.62	4.04	3.70
4	3.41	3.65	3.61	3.70
5	3.71	3.45	3.47	3.52
6a	4.01	3.84	3.91	3.86
6b	3.72	3.69	3.79	3.68
3′[g]	1.46			

[a] The chemical shift values for the signals of the N-acetate groups and for muramic acid are not listed in this table but are similar to those given in [21].
[b] Additional signals for the terminal β-ManNAc- are at C-1/H-1 100.79 ppm (164 Hz)/4.92 ppm, C-2/H-2 54.46 ppm/4.58 ppm.
[c] Additional signals for the "near to reducing end" →3)-β-ManNAc- are at C-1/H-1 101.10 ppm (163 Hz)/4.79 ppm, C-2/H-2 51.60/4.67 ppm.
[d] Additional signals for the reducing end →4)-α-GlcNAc- are at C-1/H-1 94.60 ppm (175 Hz)/5.43 ppm, C-2/H-2 54.68 ppm/3.92 ppm.
[e] Indicates the depyruvylated polysaccharide.
[f] $^{1}J_{C,H}$ coupling constants (in Hz) are shown in parentheses.
[g] Indicates the atoms of pyruvic acid (Pyr).
[h] Not determined.

In a second $^{1}H\{^{31}P\}$ decoupling difference experiment, the minor phosphorus signal could be unequivocally linked to the side chain protons 6 of a sugar residue (4.14 ppm/4.02 ppm), presumably via O-6/C-6 of muramic acid. No indications of a second minor polymer component were found in any chromatography experiment, which is in striking contradiction to the integral ratio in the ^{31}P NMR spectra. We inferred that one phosphorus signal was broadened as a consequence of slow molecular dynamics in an anisotropic environment, *e.g.*, by being bound to a micelle, while the other phosphorus nucleus was still exposed to a more mobile molecular environment. To test this hypothesis, a sample of the SCWP-peptidoglycan complex of *P. alvei* CCM 2051 was treated with SDS and EDTA and sonicated. This procedure, according to Strain *et al.* [32], very efficiently opens micelles of lipopolysaccharides in aqueous solutions. In spectra taken immediately (approximately 5 min) after this treatment the ^{31}P integral ratio of the two ^{31}P signals shifted almost to 1:1, while the linewidth of the higher frequency signal decreased significantly. After about 3 h, the line had broadened again and the integral ratio returned to approximately 1:5, indicating the formation of new micelles. This result proves that the smaller sugar-6-phosphate signal does not originate from a second, unrelated minor component.

These NMR-experiments have to be interpreted together with the fact, that there is no spectroscopic indication for an extra sugar residue between the reducing end GlcNAc of the repeating unit and the pyrophosphate group of the SCWP. This allowed to postulate that in the polymer of *P. alvei* CCM 2051 the first oligosaccharide unit is linked with an α-GlcNAc residue ($^{1}J_{C,H} = 175$ Hz) to phosphate (Figure 4). Since all GlcNAc residues in the backbone of the polymer are in a β-1,3 linkage ($^{1}J_{C,H} = 164$ Hz), an inversion of the anomeric center of this terminal GlcNAc residue must have occurred.

From the combined NMR and chemical evidence the following structure for the SCWP-peptidoglycan complex, isolated from *P. alvei* CCM 2051, can be proposed: it is composed of approximately twelve identical →3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→ disaccharide repeating units with a 1:1 substitution of the ManNAc residues with pyruvate, constituting the carbohydrate portion of the SCWP-peptidoglycan complex.

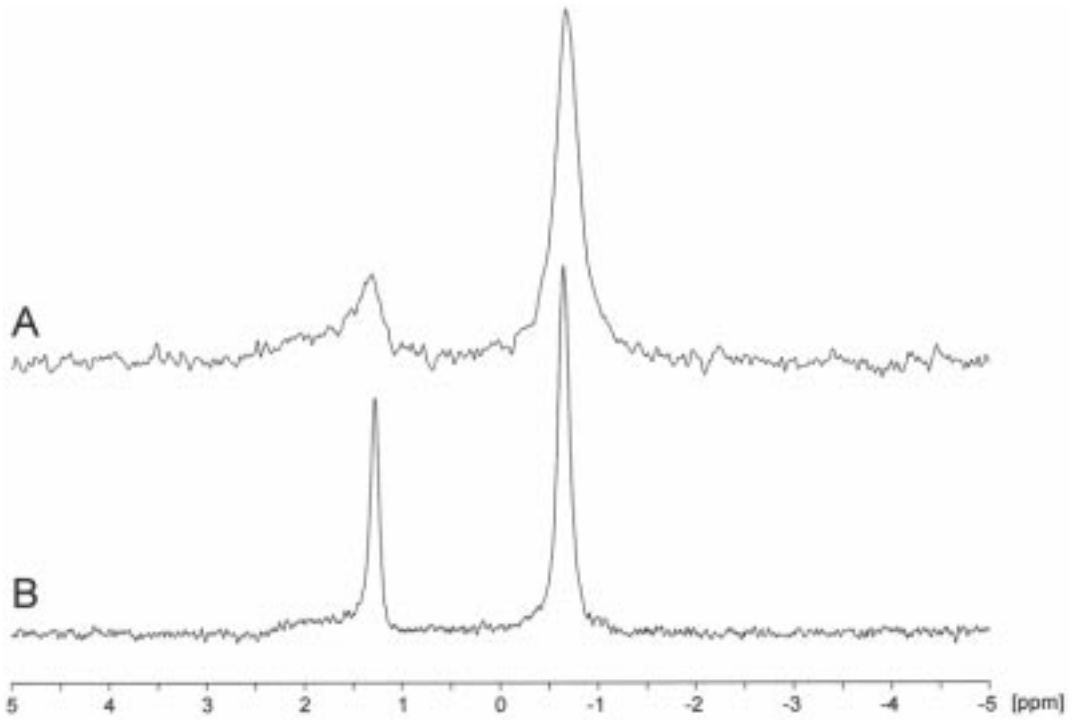

Figure 2. Comparison of the ^{31}P-NMR spectra of the SCWP-peptidoglycan comlex isolated from *P. alvei* CCM 2051 showing the significant change in the integral ratio of the two phosphorus signals. **A**, spectrum acquired without any further treatment of the sample after 22 h recording time; **B**, spectrum obtained after treatment with micelle-breaking agents (EDTA, SDS) and sonication (see text and [32]).

The unfavourable relaxation properties of the sample (both ^{31}P linewidths in excess of 30 Hz) have so far precluded the observation of a *J* coupling between the two phosphorus signals. Nevertheless, the results obtained are sufficient evidence for the presence of a pyrophosphate linkage. This assumption was further corroborated by the fact, that by sonication a reversible change of the ^{31}P signal ratio from approximately 1:10 to almost 1:1 was induced in the intact SCWP-peptidoglycan complex. It should be noted that the change in the signal integral ratio does not indicate a chemical change of the structure, rather the integral of the broad component (in the micelles) is severely underestimated by the integration procedure. The pyrophosphate bridge serves as linker between C-1 of the α-D-Glc*p*NAc residue at the reducing end of the heterosaccharide and the C-6 of muramic acid residues of the peptidoglycan layer. The peptidoglycan portion itself belongs to the A1γ type [33].

Discussion

The cell wall polysaccharides of Gram-positive bacteria can be classified into three groups according to their structural characteristics: i) teichoic acids, ii) teichuronic acids, and iii) other polysaccharides comprising neutral or acidic polysaccharides which cannot be affiliated to the two former groups (for review see [11]). The first two classes of polysaccharides are well characterized with regard to their structural compositions and linkage units. Usually, they consist of a disaccharide linkage part of varying composition, such as β-ManNAc-(1 → 4)-GlcNAc, β-ManNAc-(1 → 4)-GlcN or β-Glc-(1 → 4)-GlcNAc [13,34], and a phosphate residue which is covalently attached to the hydroxyl group at C-6 of an *N*-acetylmuramyl residue of the peptidoglycan layer (for reviews see [11,12]). Concerning the third class of cell wall polysaccharides, the carbohydrate structures of several SCWP of different *Bacillus* strains have been investigated in the past, but information on the type of linkage of these polymers to the peptidoglycan is still very limited.

In the course of the characterization of S-layer glycoprotein glycans of various *Bacillus* strains (for review see [8]), residual amounts of SCWP were always coisolated. In order to distinctively discriminate between S-layer-derived and peptidoglycan-associated polysaccharides, the mode of association between these SCWP and the peptidoglycan layer was identified in the respective bacilli, carrying glycosylated S-layers. The first SCWP-peptidoglycan complex, whose structure was completely elucidated on this context, is the polymer of *B. stearothermophilus* NRS 2004/3a [21]. This anionic polymer, comprising on average five tetrasaccharide repeating units with the structure → 4)-β-D-ManA2,3$(NAc)_2$-(1 → 6)-α-D-Glc-(1 → 4)-β-D-ManA2,3$(NAc)_2$-(1 → 3)-α-D-GlcNAc-(1 → , represents the SCWP of *B. stearothermophilus* wild-type strains. A pyrophosphate bridge was identified by 600 MHz NMR spectroscopy to be the linker between the

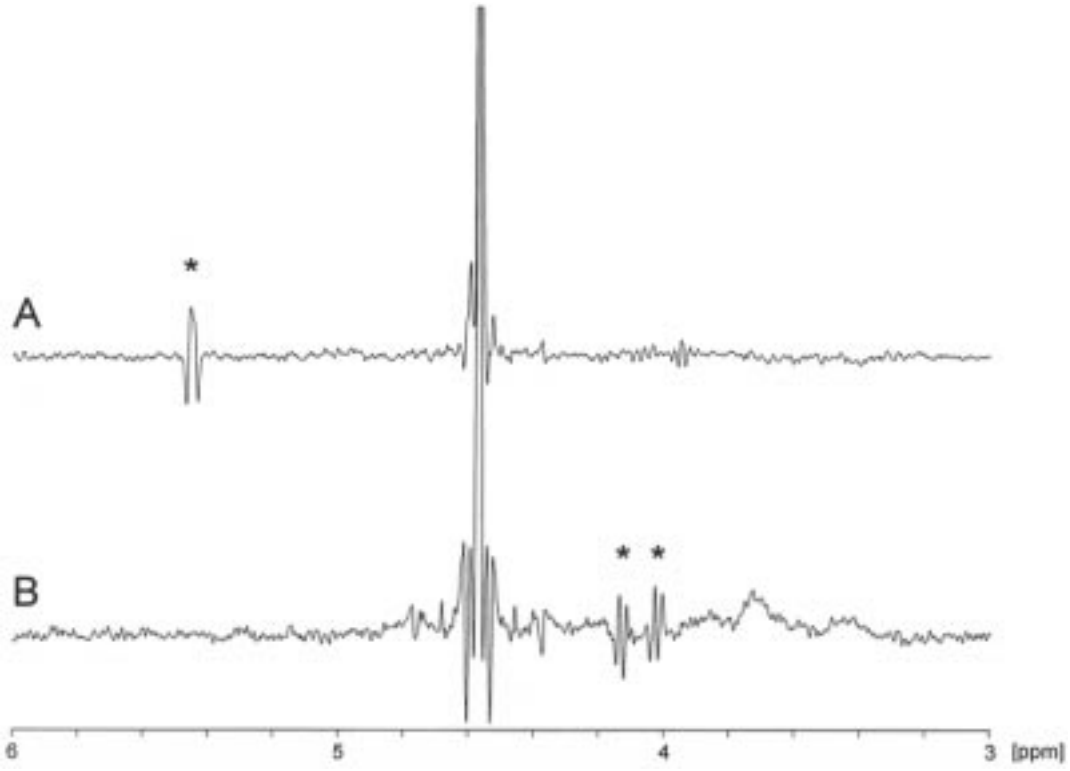

Figure 3. $^{1}H\{^{31}P\}$ decoupling difference NMR spectra of the SCWP-peptidoglycan complex isolated from *P. alvei* CCM 2051. **A**, difference spectrum decoupled at a ^{31}P shift of -0.69 ppm; **B**, difference spectrum decoupled at a ^{31}P shift of 1.33 ppm. The ^{1}H signals appearing upon decoupling are marked with an asterisk.

glycan portion and the peptidoglycan component, thus allowing a classification of this polymer into group iii) of the scheme of Araki and Ito [11]. In this present study, the SCWP-peptidoglycan complex of *P. alvei* CCM 2051 was characterized. An absolute requirement for this approach was the isolation of the complex in its intact form, which was ascertained from its retention behavior on the RP-HPLC column. The elution interval was comparable to that of the SCWP-peptidoglycan complex of *B. stearothermophilus* NRS 2004/3a [21]. In the *P. alvei* CCM 2051 polymer, the negative charges originate from pyruvic acid substituents which are attached to the ManNAc residues of approximately twelve $\rightarrow$3)-β-D-Man*p*NAc-(1$\rightarrow$4)-β-D-Glc*p*NAc-(1$\rightarrow$ disaccharides, constituting the entire polysaccharide chain (Figure 4). Due to the micelle formation of the SCWP-peptidoglycan sample, the connectivity between the two phosphorus atoms, linking C-1 of an α-D-Glc*p*NAc residue at the reducing end of the glycan portion and C-6 of muramic acid residues could not be demonstrated directly. However, the obtained results provide sufficient evidence for the presence of a pyrophosphate linkage.

In a previous experiment by McNeil *et al.* [35], ^{31}P NMR analysis was used to demonstrate the link between the arabinogalactan and peptidoglycan of mycobacterial cell walls. Prior to mild hydrolysis of the linkage unit -Rha*p*-(1$\rightarrow$3)-D-GlcNAc-(1$\rightarrow$*O*)-PO_2-(*O*$\rightarrow$6)-Mur- of that complex, two ^{31}P signals were observed at pH 10, one at $\delta = -1.71$, which resulted from a phosphodiester linkage and the other one at $\delta \sim 4$, indicative for a phosphomonoester group. After hydrolysis the signal at $\delta = -1.71$ completely disappeared, from which the existence of a phosphodiester linkage in the mycobacterial glycoconjugate was concluded [35]. In the *P. alvei* CCM 2051 SCWP-peptidoglycan complex, however, neither hydrolysis of the intact, RP-HPLC-purified material was performed nor have phosphorylated degradation products ever been observed. After sonication, the integral ratio of the ^{31}P-signals shifted reversibly from about 1:10 to almost 1:1 and, after about three hours back to about 1:5. Since the chemical shifts of both ^{31}P signals remained in the phosphodiester region [21], those data were taken as a strong support for the presence of a pyrophosphate linkage in the SCWP-peptidoglycan complex of *P. alvei* CCM 2051. The fact that only one of the two pyrophosphate signals is broadened in such a significant way deserves special attention in future investigations.

Interestingly, an inversion of the anomeric configuration of the terminal D-GlcNAc residue was observed. A similar phenomenon of inversion was reported to occur in the coreless S-layer glycoprotein glycan of *T. thermosaccharolyticum* D120-70 [36]. Only recently, comparable findings concerning a change of the anomeric configuration of the reducing end sugar in polysaccharide chains have been described for the LPS

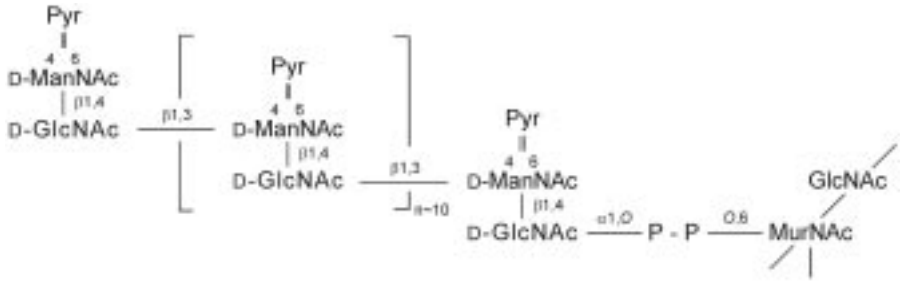

Figure 4. Structure of the SCWP-peptidoglycan complex isolated from *P. alvei* CCM 2051.

O-antigens of *Pseudomonas aeruginosa* [37] and *Salmonella enterica* [38]. Even in the latter reports there are only speculations about the precise reaction mechanism. In these cases, it is hypothesized that the linking sugar is in α-configuration during polymerization of the lipid-bound O-chain repeating unit and is then inverted during ligation to either the core region or an interlinking unit. It is assumed that the attachment of the first repeating unit is a critical biosynthetic step for the complete and correct elongation of the O-chain polysaccharide. In order to substantiate the biosynthetic and biological relevance of these observations, further investigations will be required. From preliminary mass spectrometry data of lipid-activated biosynthetic intermediates of the SCWP of *Aneurinibacillus thermoaerophilus* DSM 10155 [Graninger, Schäffer, Peter-Katalinić, Messner, unpublished observations] it seems possible that these polymers are fully assembled in the cytoplasm and transferred across the cytoplasmic membrane in the lipid-bound stage. Whether the inversion of the anomeric configuration of the linkage sugar occurs before or after the transfer of the polysaccharide chain to the lipid carrier, or even during the establishment of the linkage to specific *N*-acetylmuramyl residues is not yet known.

In contrast to the phosphodiester linkage which is typical for teichoic and teichuronic acid-like structures [12], the SCWP of *B. stearothermophilus* NRS 2004/3a and *P. alvei* CCM 2051 are bound directly, without a linkage unit disaccharide, via a pyrophosphate bridge to the peptidoglycan sacculus. An indication for the presence of the same type of linkage was also obtained for the SCWP of *B. sphaericus* CCM 2177 after treatment with 40% HF for 48 h [16]. In this case, the putative pyrophosphate bridge was split and the SCWP was liberated from the peptidoglycan. In addition, the phosphate residue of the polysaccharide portion was also cleaved off, leaving a free sugar residue at the reducing end. Based on the data set for the SCWP-peptidoglycan complex of *P. alvei* CCM 2051 and NMR reference data [14,39–41], the proton/carbon signals observed for the reducing end sugar of this HF-degradation product at 5.21 ppm/91.8 ppm should have been interpreted as α-GlcNAc instead of reducing end ManNAc. In addition, the signals found at 4.88 ppm/100.2 ppm are typical for terminal β-ManNAc. The signal of the pyruvylated terminal D-ManNAc unit was observed at 4.92 ppm/100.8 ppm. Although the signals for H-3/C-3 of that ManNAc, non-substituted in position 3, cannot be determined unambiguously, the missing β-shift at the signals of H-2/C-2, observed at 4.58 ppm/54.46 ppm, indicates position 3 not to be glycosidated.

In the SCWP of *P. alvei* CCM 2051 (this study) and *B. sphaericus* CCM 2177 [16] identical disaccharide motifs were found and the anionic character of both polymers resides with its pyruvic acid residues. Concerning HF-treatment for the liberation of the SCWP from the peptidoglycan portion, with the neutral SCWP of *T. thermosaccharolyticum* E207-71 [Schäffer, Messner, unpublished observations] a repeating unit-wise degradation of the polymer was observed, implying that the full-length polymer cannot be recovered after this treatment. The molecular masses of the SCWP of *P. alvei* CCM 2051, *B. stearothermophilus* NRS 2004/3a and *T. thermosaccharolyticum* E207-71 were determined with the polymer still attached to the peptidoglycan portion, giving molecular masses in the range of 4000 to 6500 Da.

In general, these polymers constitute a substantial proportion of the Gram-positive peptidoglycan sacculus, implicating that they cannot only be of secondary relevance. The amount varies between 20% and 10%, as determined for the HF-extracted SCWP of *B. stearothermophilus* PV72/p2 [18] and for the HPLC-purified SCWP-peptidoglycan complex of *P. alvei* CCM 2051, respectively. In the peptidoglycan of *B. stearothermophilus* NRS 2004/3a, 20–25% of the muramic acid residues are substituted with the SCWP of that organism [21]. Whether the substitution occurs in a regular or in a random manner has not been determined.

The relevance of anionic polymers for the bacterial cell is frequently discussed in context with contributing to the polyelectrolyte gel structure of the wall and maintaining the peptidoglycan network in an expanded state, caused by charge repulsion [42]. Since there is strong evidence for the presence of neutral SCWP in various S-layer glycoprotein-covered Bacillaceae, *e.g.*, *T. thermosaccharolyticum* strains E207-71 and D120-70 [Schäffer, Kählig, Messner, unpublished observations] and *A. thermoaerophilus* DSM 10155 [Wugeditsch, Schäffer, Messner, unpublished observations], functions other than charge-based phenomena have to be assumed for these SCWP. Functional studies of SCWP of different *B. stearothermophilus* PV72 strains have demonstrated that they are involved in the attachment of the respective S-layer proteins to the peptidoglycan layer of these organisms [18–20].

In fact, the SCWP of *B. stearothermophilus* PV72/p2 was demonstrated to be responsible for anchoring the S-layer subunits via the N-terminal part to the peptidoglycan sacculus [18,20].

From the compositional and structural data we conclude that the pyrophosphate linkage is the preferred type of covalent linkage between the SCWP and the peptidoglycan in S-layer covered, Gram-positive organisms. The polymer structures elucidated so far, include anionic and neutral ones. Thus we suggest their classification into the group of polymers differing from teichoic and teichuronic acids, corresponding to group iii) of Araki and Ito [11].

Concerning the immunological relevance of SCWP-peptidoglycan complexes copurified with S-layer preparations, which serve as immobilisation matrices for the construction of model conjugate vaccines (for review see [1,43]), it can be assumed that the muramyldipeptide portion rather than the specific structure of the SCWP is immunologically active. It seems plausible that the disaccharide motifs found in the SCWP have additional, immunostimulatory activity [44]. There are also presumptions that the increased activity is due to the high affinity of the GlcNAc moiety for the macrophage mannose receptor [45]. Thus, sugar-muramyldipeptide derivatives might be seen as immunomodulators in humans, with disaccharide derivatives being superior to monosaccharide derivatives [46]. This concept supports the definition of S-layers as immobilisation matrices with intrinsic adjuvant properties [47] and makes them even more promising carrier systems for future vaccine development.

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