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Structural and Functional Analyses of the Secondary Cell Wall Polymer of *Bacillus sphaericus* CCM 2177 That Serves as an S-Layer-Specific Anchor

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Sacculi of *Bacillus sphaericus* CCM 2177 contain a secondary cell wall polymer which was completely extracted with 48% hydrofluoric acid. Nuclear magnetic resonance analysis showed that the polymer is composed of repeating units, as follows: →3)-[4,6-*O*-(1-carboxyethylidene)]$_{\sim 0.5}$-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→. The N-terminal part of the S-layer protein carrying S-layer homologous motifs recognizes this polymer as a binding site.

Crystalline bacterial cell surface layers (S-layers) represent the outermost cell envelope component of many bacteria and archaea (for reviews, see references 4, 23, 27, and 28). S-layers are composed of identical proteinaceous subunits, and they assemble into either oblique, square, or hexagonal lattice types. To answer the question of how S-layer proteins of gram-positive bacteria are anchored to the rigid cell wall layer, the whole cell envelope complexes of *Bacillus stearothermophilus* wild-type strains and an oxygen-induced variant strain were analyzed, and secondary cell wall polymers (SCWP) were found to function as binding sites for this class of secreted proteins (9, 21). Based on structure, teichoic acids, teichuronic acids, lipoteichoic acids, and lipoglycans are distinguished among SCWP (for reviews, see references 3, 10, 19, and 22). Most of the biological functions ascribed to SCWP, such as binding of cations, protecting the cell against toxic metals, keeping the peptidoglycan sacculus in an expanded state by charge repulsion, binding of protons to create an acidic cell wall during bacterial growth, and providing a biophysical barrier to prevent diffusion of substances, have been viewed in the context of their acidic nature (for a review, see reference 3).

In contrast to those of *B. stearothermophilus* wild-type strains (9, 11), the S-layer proteins of most gram-positive bacteria carry three typical S-layer homologous (SLH) motifs (16) at the N-terminal part, each of them consisting of approximately 50 to 60 amino acids. In addition to being present in S-layer proteins, SLH motifs were also identified at the C-terminal end of cell-associated exoenzymes or other exoproteins (14, 17). In several studies, SLH motifs were found to anchor the different types of cell-associated exoproteins to the rigid cell wall layer (6, 7, 14, 15, 17, 18, 20). However, only for a few organisms was it confirmed that an SCWP is involved in the binding process (6, 7, 18, 21). In the present study, the structure of the SCWP of *Bacillus sphaericus* CCM 2177 was characterized by nuclear magnetic resonance (NMR) analysis. Moreover, evidence was provided that the SCWP, not the peptidoglycan, recognizes the N-terminal part of the S-layer protein carrying at least one SLH motif.

Characterization of the S-layer of *B. sphaericus* CCM 2177. Freeze-etching of whole cells from *B. sphaericus* CCM 2177 grown in continuous culture in nutrient broth at 30°C at a dilution rate of 0.16 h^{-1} revealed that the cell surface was completely covered with a square S-layer lattice showing a rather smooth outer surface (data not shown). The S-layer protein could completely be extracted from cell wall fragments prepared as described previously (9, 21, 25) with 5 M guanidine hydrochloride (GHCl). During removal of GHCl by dialysis against 10 mM $CaCl_2$ at 20°C, the S-layer subunits assembled into flat sheets with a maximum width of 2 μm. Negative staining and freeze-drying revealed that the self-assembly products represented double layers in which the individual S-layers were oriented to each other, with corrugated inner surfaces (Fig. 1). Upon dialysis of the GHCl-extracted S-layer protein against distilled water or 10 mM EDTA at 20°C, mainly amorphous aggregates were observed in negatively stained preparations (data not shown). The positive effect of calcium ions on the in vitro self-assembly was also seen by the degree of assembly (percentage of total S-layer protein assembled [21]). When dialysis was performed against distilled water or 10 mM EDTA, the degree of assembly was <5% after 4 h and increased to 25% 18 h after the dialysis procedure was started. For comparison, degrees of assembly of 55 and 80%, respectively, were achieved at the above-mentioned points of time when dialysis was performed against 10 mM $CaCl_2$.

Chemical analyses of native and HF-extracted peptidoglycan-containing sacculi. Native peptidoglycan-containing sacculi and those extracted with 48% HF were prepared as described previously (21). The results from amino acid and amino sugar analyses of hydrolyzed samples of native and HF-extracted sacculi are summarized in Table 1. For calculating the molar ratios between the individual components, glutamic acid (Glu) was set to a value of 1. With the exception of the excess glucosamine ($GlcNH_2$) and the occurrence of substantial amounts of mannosamine ($ManNH_2$), the molar ratios of all other components were typical of the A4α-chemotype (26). Extracting the native peptidoglycan-containing sacculi with 48% HF for 48 h at 4°C (21) led to the complete removal of the excess of $GlcNH_2$ and of $ManNH_2$, whereas the molar ratios for all other peptidoglycan constituents remained unchanged

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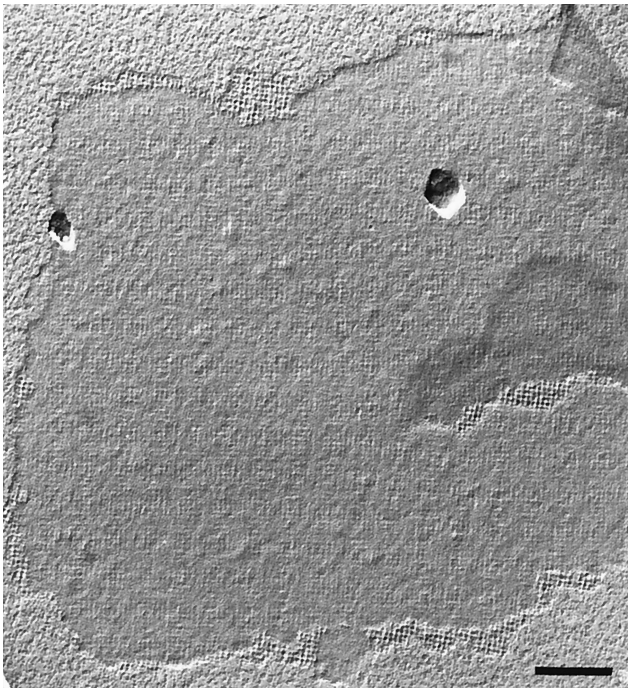

FIG. 1. Electron micrograph of a freeze-dried S-layer self-assembly product of *B. sphaericus* CCM 2177. The outer S-layer surface is rather smooth, whereas the inner S-layer surface is much more corrugated. Bar, 200 nm.

(Table 1). In comparison to native peptidoglycan-containing sacculi, those extracted with HF did not show any changes in size and morphology but appeared less electron dense in negatively stained preparations (Fig. 2a and d).

Chemical and NMR analysis of the HF-extracted purified SCWP. The HF-extracted SCWP, which represented about 50% of the native peptidoglycan-containing sacculi by weight, was purified by gel permeation chromatography according to a method described previously (24). Hydrolysis of the SCWP with 4 N HCl (6 h at 110°C) led to a $GlcNH_2$-to-$ManNH_2$ molar ratio of 1 to 1, whereas a molar ratio of 2.1 to 1 was obtained by using 6 N HCl (6 h at 110°C). For NMR analysis, 4.6 mg of the purified SCWP was dissolved in D_2O (0.6 ml, 99.95%). Spectra were recorded at 300 and 330 K at 300.13 MHz for ^1H and at 75.47 MHz for ^{13}C with a Bruker *AVANCE 300* spectrometer equipped with a 5-mm QNP probehead with z gradients. ^1H spectra were referenced internally to sodium 3-trimethylsilyl-1-propane sulfonate ($\delta = 0$); ^{13}C spectra were referenced externally to 1,4-dioxane ($\delta = 67.40$). COSY, TOCSY, HMQC, HMBC, and NOESY spectra were recorded with standard XWINNMR software (Bruker). ^1H and ^{13}C NMR spectra recorded at 300 K revealed the presence of pyruvate groups as indicated by a signal at 1.50 ppm in the proton domain and by signals at 24.79, ~100.0, and 174.9 ppm in the HMBC spectrum. The signal intensity of the pyruvate methyl groups corresponded to ~20 to 25% of the integral of the neighboring N-acetyl groups at δ 2.02 to 2.04. Pyruvate substituents were cleaved off, and a well-resolved spectrum was recorded at 330 K (Table 2). The ^1H NMR spectrum displayed two major signals for anomeric protons at 4.82 ppm ($J \sim 1.0$ Hz) and 4.59 ppm ($J \sim 7.7$ Hz) as well as two minor signals at 4.88 and 5.21 ppm. Since the ^{13}C NMR spectrum contained only two major signals for anomeric carbons (100.55 and 98.89 ppm), the SCWP had to be composed of disaccharide repeating units occurring in the β-anomeric configuration ($J_{C1} = 164.2$ and 165.1 Hz, respectively). The two minor anomeric ^1H and ^{13}C signals were assigned to reducing α-configured N-acetyl mannosamine (ManNAc; 5.21 and 91.8 ppm) and terminal 2-acetamido-2-deoxy-β-D-hexopyranosyl units (4.88 and 100.2 ppm). Comparison of the proton signal intensities indicated an average of 8 to 9 disaccharide residues in the polysaccharide sample. This finding was substantiated by matrix-assisted laser desorption ionization–time of flight data which revealed major signals at 2,073.4, 2,887.2, 3,294.2, 3,700.9 and 4,107.5 mass units, corresponding to 6 to 10 *N*-acetyl hexosamine disaccharide units [$(HexNAc)_2$] units ($M + Na + H_2O$).

Homonuclear and heteronuclear correlation spectra allowed the straightforward assignment of two units of 2-acetamido-2-deoxy-mannopyranosyl and 2-acetamido-2-deoxy-glucopyranosyl residues. NOESY spectra revealed an interresidue Noe from H-1 of the *N*-acetyl glucosamine (GlcNAc) residues to H-3 of the ManNAc units, whereas irradiation of H-1 of the ManNAc moieties yielded signal enhancement of H-3 and -4 (having similar chemical shifts) of the GlcNAc units. Since substitution at C-3 would lead to an upfield shift of C-2, the observed shift value for C-2 (56.14 ppm) was only compatible with a 4-O-substitution of the GlcNAc unit. The observed value for the optical rotation $[\alpha]_D^{20} - 16°$ (*c* 0.4, H_2O) of the SCWP indicated the presence of two units each of β-D-configured residues of ManNAc and GlcNAc (12). Thus, the structure of the pyruvic acid-free SCWP may be proposed to be as follows: →3)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→.

Recrystallization and affinity studies with whole S-layer protein and proteolytic cleavage fragments as well as native and HF-extracted peptidoglycan-containing sacculi. The GHCl-extracted S-layer protein was mixed with native or HF-extracted sacculi under conditions described previously (21), and the suspensions were dialyzed against distilled water, 10 mM $CaCl_2$, or 10 mM EDTA at 4°C for 18 h. Ultrathin sectioning revealed that complete outer and inner S-layers were formed on native peptidoglycan-containing sacculi when dialysis was performed against distilled water or 10 mM $CaCl_2$ (Fig. 2c). Negative staining showed that the formation of an extensive square lattice structure was strongly dependent on the presence of calcium ions during dialysis (Fig. 2a and b). If dialysis was against distilled water, only small, randomly oriented patches with square lattice symmetry consisting of up to 10 morphological units were formed (data not shown). When dialysis was performed against 10 mM EDTA, neither the square S-layer lattice nor the outer or inner S-layer could be observed (data not shown). Thus, the results from electron microscopic investigations demonstrated that calcium ions are required for the correct binding of the S-layer protein to the

TABLE 1. Composition of native and HF-extracted peptidoglycan-containing sacculi of *B. sphaericus* CCM 2177[a]

Amino acid or sugar	Molar composition		
	Native	HF-extracted (48 h)	HF-extracted (96 h)
$GlcNH_2$	2.76	1	0.50
Glu	1	1	1
Ala	1.33	1.29	1.36
Asp	0.51	0.52	0.53
Lys	0.53	0.54	0.54
$ManNH_2$	0.82	0	0

[a] For liberation of the peptidoglycan constituents hydrolysis was performed with 6 N HCl for 6 h at 110°C (9, 21, 24). Molar composition is shown with glutamic acid (Glu) = 1. $GlcNH_2$, glucosamine; Ala, alanine; Asp, aspartic acid; Lys, lysine; $ManNH_2$, mannosamine. (Muramic acid could not quantitatively be determined by this analysis method.)

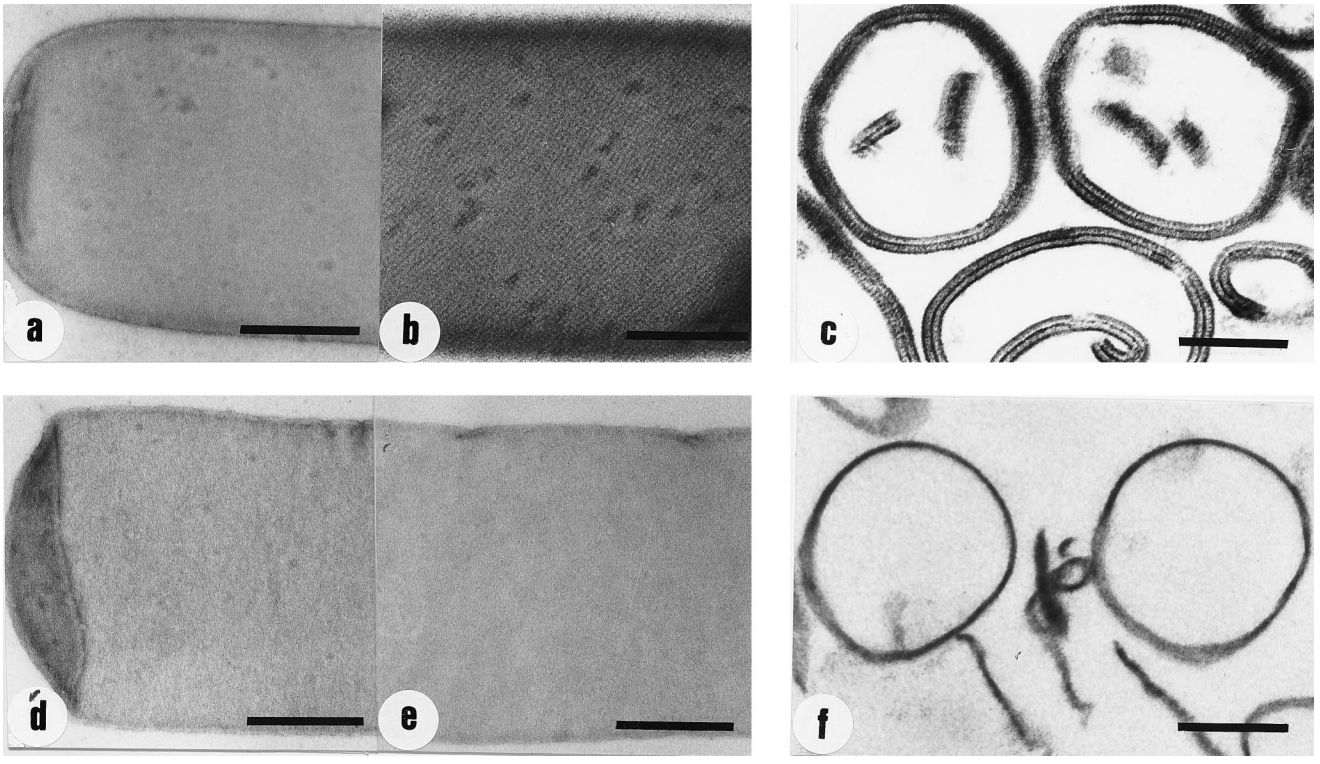

FIG. 2. Electron micrographs of negatively stained and ultrathin-sectioned preparations of native and HF-extracted peptidoglycan-containing sacculi of *B. sphaericus* CCM 2177. (a and b) Negatively stained preparations of native peptidoglycan-containing sacculi before and after recrystallization of the S-layer protein, respectively; (c) ultrathin-sectioned preparation of native peptidoglycan-containing sacculi after recrystallization of the S-layer protein; (d and e) negatively stained preparations of HF-extracted peptidoglycan-containing sacculi (48% HF, 48 h at 4°C) before and after the addition of the GHCl-extracted S-layer protein and dialysis, respectively; (f) ultrathin-sectioned preparation of HF-extracted peptidoglycan-containing sacculi after the addition of the GHCl-extracted S-layer protein and dialysis. Bars, 250 nm.

rigid cell wall layer as well as for the formation of the square lattice structure. Independent of the dialysis conditions, the S-layer protein did not bind to HF-extracted (48% HF, 48 h, 4°C) sacculi (Fig. 2d to f), which, according to chemical analysis, represented pure peptidoglycan (Table 1).

S-layer self-assembly products were dissolved in 2 M GHCl, and the S-layer protein was cleaved with endoproteinase Glu-C under conditions described previously (9, 21, 24). After removing GHCl by dialysis against 50 mM Tris-HCl buffer (pH 7.2), native peptidoglycan-containing sacculi were added. When the mixture was centrifuged, uncleaved S-layer protein and three major protein bands with estimated relative molecular masses of 57,000, 38,000, and 32,000 Da were enriched in the pellet (Fig. 3), whereas all other cleavage fragments remained unbound. The protein bands which showed affinity to native sacculi had N termini (AQVND) identical to that of the whole S-layer protein (AQVNDYNKISGYAKEAVQSLVDQGVIOGDTNGNFN) (SLH motif underlined) showing an estimated

TABLE 2. NMR data of the pyruvate-free SCWP of *B. sphaericus* CCM 2177 recorded at 330 K[a]

Atom position	NMR analysis results for:					
	→3)-β-ManNAc			→4)-β-GlcNAc		
	^{1}H		^{13}C chemical shift (ppm)	^{1}H		^{13}C chemical shift (ppm)
	Chemical shift (ppm)	*J* (Hz)		Chemical shift (ppm)	*J* (Hz)	
1	4.82	1.0	100.55	4.59	7.8	98.89
2	4.65	4.3	50.81	3.74	ND	56.14
3	4.03	9.8	77.99	3.70	ND	73.40
4	3.61	9.6	66.03	3.69	ND	80.06
5	3.46	2.4	77.25	3.52	ND	75.46
6	3.90, 3.78	12.2, 5.0	61.62	3.86, 3.80	ND	61.27

[a] Other signals were as follows: 2.02 and 2.04 ppm (CH_3), 23.38 and 23.07 ppm (CH_3), and 175.31 and 175.41 ppm (CO). ND, not determined.

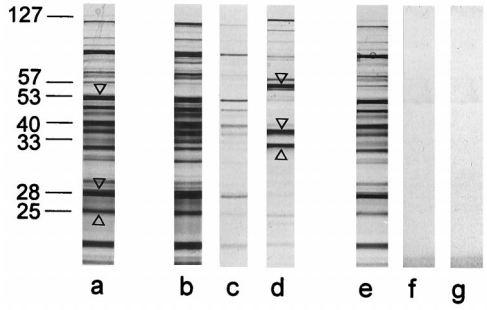

FIG. 3. Lanes: a, SDS-PAGE pattern obtained by proteolytic degradation of the whole S-layer protein of *B. sphaericus* CCM 2177 with endoproteinase Gluc-C; b and e, proteolytic cleavage fragments that remained in the clear supernatant after incubation with native and HF-extracted peptidoglycan-containing sacculi, respectively; c and f, proteolytic cleavage fragments which could be removed from the bound fraction by washing native and HF-extracted peptidoglycan-containing sacculi, respectively, with buffer; d and g, proteolytic cleavage fragments which remained attached to native and HF-extracted peptidoglycan-containing sacculi, respectively. Molecular masses (in thousands) are shown at the left. Protein bands subjected to N-terminal sequencing are indicated by arrowheads.

relative molecular mass of 127,000. Distinct protein bands of those remaining in the clear supernatant showing apparent relative molecular masses of 53,000, 28,000, and 25,000 Da were also subjected to N-terminal sequencing. Their N-terminal regions were TAPNG, DVKNT, and TAPNG, none of which could be identified within the N-terminal sequence of the whole S-layer protein. None of the proteolytic cleavage fragments could bind to HF-extracted sacculi (Fig. 3) representing pure peptidoglycan (Table 1).

To conclude, the N-terminal part of the S-layer protein from *B. sphaericus* CCM 2177, carrying at least one SLH motif, recognizes a net negatively charged SCWP composed of GlcNAc, ManNAc, and pyruvic acid as the binding site. According to the chemical composition, this SCWP should be attributed to the teichuronic acids (3). The basic structure of the SCWP of *B. sphaericus* CCM 2177 is similar to those of polysaccharides from *Bacillus polymyxa* AHU 1385 (13), type e capsular polysaccharide of *Haemophilus influenzae* (29), and glycans copurified with the S-layer glycoproteins of *Thermoanaerobacterium thermosaccharolyticum* E207-71 and D120-70 (1, 2). The N-terminal 35 amino acids of the S-layer protein from *B. sphaericus* CCM 2177 showed 97.1 and 88.6% identity to the corresponding N-terminal parts of the S-layer proteins from *B. sphaericus* P-1 and 2362 (5, 8). The S-layer proteins from *B. sphaericus* P-1 and 2362 showed 80% identity for their N-terminal regions, while the sequence identity beyond the N-terminal 200 amino acids was less than 20%. Accordingly, the internal cleavage fragments of the S-layer protein of *B. sphaericus* CCM 2177 could not be mapped on the sequences of the S-layer proteins from *B. sphaericus* P-1 and 2362.

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