High-Affinity Interaction between the S-Layer Protein SbsC and the Secondary Cell Wall Polymer of *Geobacillus stearothermophilus* ATCC 12980 Determined by Surface Plasmon Resonance Technology[†]

Judith Ferner-Ortner,¹ Christoph Mader,² Nicola Ilk,¹ Uwe B. Sleytr,¹ and Eva M. Egelseer^{1*}

Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences, A-1180 Vienna,¹ and Bio-Products and Bio-Engineering Aktiengesellschaft/Biomedizinische Forschungsgesellschaft mbH, A-1090 Vienna,² Austria

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Surface plasmon resonance studies using C-terminal truncation forms of the S-layer protein SbsC (recombinant SbsC consisting of amino acids 31 to 270 [rSbsC_{31–270}] and rSbsC_{31–443}) and the secondary cell wall polymer (SCWP) isolated from *Geobacillus stearothermophilus* ATCC 12980 confirmed the exclusive responsibility of the N-terminal region comprising amino acids 31 to 270 for SCWP binding. Quantitative analyses indicated binding behavior demonstrating low, medium, and high affinities.

Composed of a single protein or glycoprotein species, bacterial cell surface layers, or S-layers, represent the simplest biological membrane developed during evolution (26, 27). These crystalline arrays, which are characteristic of many bacteria and most archaea, assemble into lattices with either oblique, square, or hexagonal symmetry (1, 23, 27, 30, 31, 33). Even after isolation from the cell wall, S-layer proteins frequently maintain the ability to self-assemble in suspensions or to recrystallize on peptidoglycan-containing sacculi (PGS), at the air-water interface, or on artificial solid supports, which is essential for many nanobiotechnological applications (28, 29, 32, 33).

At least for S-layer proteins of *Bacillaceae*, the N-terminal region was found to be responsible for anchoring the S-layer subunits to the underlying rigid cell envelope layer by binding to a heteropolysaccharide termed the secondary cell wall polymer (SCWP) (19, 21, 24). In *Bacillaceae*, at least two types of mechanisms for the binding of S-layer proteins and SCWPs have been identified. The first one involves so-called S-layer homologous (SLH) domains and pyruvylated SCWPs (3, 4, 8, 9, 14–17, 19–22) and was previously found to be widespread among prokaryotes (16). By using surface plasmon resonance (SPR) spectroscopy, it has been demonstrated that the SLH domain of the S-layer protein SbsB of *Geobacillus stearothermophilus* PV72/p2 shows specific affinity for the pyruvylated SCWP of this organism but that it does not bind to any other type of SCWP or to peptidoglycan (PG) or other heteropolysaccharides (15).

The second type of binding mechanism for S-layer proteins and SCWPs has been identified in the *G. stearothermophilus* wild-type strains PV72/p6, NRS 2004/3a, and ATCC 12980 (6, 11, 12, 24, 25), as well as a temperature-derived strain variant of the latter (5). *G. stearothermophilus* ATCC 12980 produces

the S-layer protein SbsC, the subject of the study described here. This binding mechanism involves an SCWP that consists of *N*-acetylglucosamine (GlcNAc), glucose, and 2,3-dideoxy-diacetamido mannosamine uronic acid in the molar ratio of 1:1:2 (24) and a highly conserved N-terminal region that does not comprise an SLH domain (5, 6, 11, 12). The mature S-layer protein SbsC of *G. stearothermophilus* ATCC 12980 comprises amino acids 31 to 1099 and self-assembles into an oblique lattice type (12). The production of different truncated forms of SbsC indicated that the N-terminal part comprising amino acids 31 to 257 is responsible for cell wall binding (11).

In the present study, for the first time, quantitative SPR analyses were performed in order to investigate the basic interaction in anchoring an S-layer protein devoid of SLH motifs to the rigid cell wall layer.

Cloning and expression of genes encoding the different SbsC truncation constructs and isolation and purification of the respective proteins. Two C-terminal recombinant SbsC (rSbsC) truncation forms (rSbsC comprising amino acids 31 to 270 [rSbsC_{31–270}] and rSbsC_{31–443}) and one N-terminal rSbsC truncation form (rSbsC_{638–1099}) were constructed by PCR amplification of the corresponding DNA fragments from the chromosomal DNA of *G. stearothermophilus* ATCC 12980 as described in reference 12 by using AccuPrime Pfx DNA polymerase (Invitrogen) and the primers listed in Table 1, which introduced the *Nco*I and *Xho*I restriction sites at the 5' and 3' ends, respectively. The digested PCR products were ligated with plasmid pET28a+, cloned into *Escherichia coli* TG1, and expressed in OneShot *E. coli* BL21 Star(DE3) cells as described in reference 11. In *E. coli* samples induced by isopropyl- β -D-thiogalactopyranoside (IPTG) to express the different sbsC derivatives, the apparent molecular masses of the protein bands on sodium dodecyl sulfate (SDS) gels corresponded well to the calculated molecular masses of the SbsC truncation constructs (Table 2). The final identification of the protein bands as the N- or C-terminal SbsC truncation forms was accomplished by N-terminal sequencing (Table 2).

As shown by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 1), the rSbsC truncation constructs rSbsC_{31–270}, rSbsC_{31–443}, and rSbsC_{638–1099}, which did not carry any affinity

* Corresponding author. Mailing address: Center for NanoBiotechnology, University of Natural Resources and Applied Life Sciences, Gregor Mendel-Strasse 33, A-1180 Vienna, Austria. Phone: 43-1-47 654 2233. Fax: 43-1-478 91 12. E-mail: eva-maria.egelseer@boku.ac.at.

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TABLE 1. Oligonucleotide primers used for PCR amplification of the gene sequences encoding the various truncated rSbsC forms

rSbsC form	Primer designation	Primer sequence ^a (5' to 3') or reference
rSbsC ₄₄₇₋₁₀₉₉		11
rSbsC ₆₃₈₋₁₀₉₉	NcoI-rSbsCaa638-forw XhoI-rSbsC-aa1099-rev	<u>CGGAATT</u> CCATGG TAAAGTACCTGTAAATGTAGTTGC <u>GACCG</u> CTCGAG <u>T</u> TACGATGCTGATTTTGTACCAATTG
rSbsC ₃₁₋₂₇₀	NcoI-rSbsCaa31-forw XhoI-rSbsC-aa270-rev	<u>CGGAATT</u> CCATGG CAACGGACGTGGCGAC <u>GACCG</u> CTCGAG <u>T</u> TAAATCGCACTTACACTCTCAACC
rSbsC ₃₁₋₄₄₃	NcoI-rSbsCaa31-forw XhoI-rSbsC-aa443-rev	<u>CGGAATT</u> CCATGG CAACGGACGTGGCGAC <u>GACCG</u> CTCGAG <u>T</u> TAAATCCTCAACTGCAAGGATTACG

^a Underlining, boldface, and double underlining indicate overhangs, restriction sites, and start or stop codons, respectively.

tag, could be isolated from the soluble cytoplasmic fraction of OneShot *E. coli* BL21 Star(DE3) cells by ammonium sulfate precipitation and purified by gel permeation chromatography according to the procedure described by Jarosch et al. (11).

Investigation of the affinities of the various N- or C-terminal rSbsC truncation forms for PGS and PG. The preparation of cell wall fragments, the isolation of PGS, and the extraction of the SCWP were performed as described in previous studies (6, 7, 19). By treating PGS with hydrofluoric acid (48%) for 40 h at 4°C, pure PG of the A1 γ chemotype, as well as the isolated SCWP, could be obtained (19).

To investigate the affinities of the truncated rSbsC forms for native PGS, as well as for PG of the A1 γ chemotype devoid of the SCWP in qualitative terms, 500- μ g samples of lyophilized rSbsC₃₁₋₂₇₀, rSbsC₃₁₋₄₄₃, rSbsC₄₄₇₋₁₀₉₉, and rSbsC₆₃₈₋₁₀₉₉ were dissolved in 0.7 ml of 5 M guanidine hydrochloride in 50 mM Tris-HCl buffer (pH 7.2). After dialysis against 50 mM Tris-HCl buffer (pH 7.2) for 18 h at 4°C and centrifugation (36,000 $\times$ *g* for 20 min at 4°C), the concentrations of the soluble proteins at 280 nm were determined. For affinity studies, 70- μ g samples of the soluble rSbsC truncation forms rSbsC₃₁₋₂₇₀, rSbsC₃₁₋₄₄₃, rSbsC₄₄₇₋₁₀₉₉, and rSbsC₆₃₈₋₁₀₉₉ were incubated with 150 μ g of PGS or PG in 600 μ l of 50 mM Tris-HCl buffer (pH 7.2), and the mixtures were stirred for 30 min at room temperature. After centrifugation (36,000 $\times$ *g* for 20 min at 4°C), followed by three washing steps, pellets and supernatants were analyzed by SDS-PAGE. The precipitation of the proteins themselves could be excluded, since none of the rSbsC forms were detected in the pellet (Fig. 2, lanes a, d, g, and j). The N-terminally truncated forms rSbsC₄₄₇₋₁₀₉₉ and rSbsC₆₃₈₋₁₀₉₉ revealed no affinity for native PGS (Fig. 2, lanes h and k) or for pure PG (Fig. 2, lanes i and l). In contrast, the C-terminal truncation constructs rSbsC₃₁₋₂₇₀ and rSbsC₃₁₋₄₄₃ could bind to PGS (Fig. 2, lanes b and e), indicating that the

N-terminal part of SbsC comprises the binding region for either PG or the SCWP but has no affinity for pure PG (Fig. 2, lanes c and f), which means that the SCWP is the anchoring molecule. Based on these results, the C-terminal truncation constructs rSbsC₃₁₋₂₇₀ and rSbsC₃₁₋₄₄₃ were used for further quantitative SPR studies.

SPR experiments. SPR biosensor experiments were performed on a Biacore 2000 instrument essentially as described in reference 15. If not otherwise indicated, the buffer flow rate was 30 μ l/min. Experiments were generally performed by passing sample solutions over the active and reference flow cells in order to improve data quality. Surfaces were regenerated by 30-s pulses of 7 M guanidine hydrochloride in 50 mM Tris-HCl buffer (pH 7.2).

Data transformation was performed and overlay plots were prepared with BIAevaluation software. Kinetic data were analyzed in terms of the “heterogeneous ligand-parallel reactions” model as described in a previous study (15). To avoid the effects of sample dispersion, the first 10 s of the wash-on and wash-out phases was omitted from the fits (13).

SPR analyses. For quantitative analyses of the binding between rSbsC₃₁₋₂₇₀ and rSbsC₃₁₋₄₄₃, which represented the soluble analytes, and the immobilized SCWP, the SCWP of *G. stearothermophilus* ATCC 12980 was immobilized on the CM5 sensor chip after the introduction of carbonylhydrazide at its reducing end (15) by applying the standard amine coupling

TABLE 2. Survey of the properties of the recombinant truncated rSbsC forms

rSbsC form	Theoretical molecular mass (kDa)	Apparent molecular mass on SDS gels (kDa)	Calculated pI	N-terminal sequence
rSbsC ₄₄₇₋₁₀₉₉	67.091	66	4.71	AEVSE
rSbsC ₆₃₈₋₁₀₉₉	47.270	52	4.72	MVKVP
rSbsC ₃₁₋₂₇₀	26.626	26	9.09	ATDVA
rSbsC ₃₁₋₄₄₃	45.195	47	8.88	ATDVA

FIG. 1. SDS-PAGE analysis of purified truncated S-layer protein forms. Lanes: a, rSbsC₃₁₋₂₇₀; b, rSbsC₃₁₋₄₄₃; and c, rSbsC₆₃₈₋₁₀₉₉. Molecular masses in kilodaltons are given.

FIG. 2. Properties of N- or C-terminal truncation protein binding to PGS (lanes b, e, h, and k) and to PG (lanes c, f, i, and l). The soluble rSbsC forms (rSbsC₃₁₋₂₇₀, rSbsC₃₁₋₄₄₃, rSbsC₄₄₇₋₁₀₉₉, and rSbsC₆₃₈₋₁₀₉₉) were mixed with native PGS, as well as pure PG, followed by centrifugation. The proteins that bound to PG or PGS were identified by SDS-PAGE. As shown in lanes b and e, the whole amounts of rSbsC₃₁₋₂₇₀ and rSbsC₃₁₋₄₄₃ were concentrated in the pellet consisting of the native PGS, indicating that these truncation constructs could bind to PGS. In contrast, rSbsC₄₄₇₋₁₀₉₉ and rSbsC₆₃₈₋₁₀₉₉ did not bind to PGS (lanes h and k). Since the PG had no affinity for any of the rSbsC truncation proteins, none of the rSbsC forms were detected in the PG pellet (lanes c, f, i, and l). To exclude precipitation, the rSbsC forms without any cell wall component were treated in the same way as a control (lanes a, d, g, and j). Molecular masses in kilodaltons are indicated on the left.

method according to the manufacturer's protocol (2). To provide a low-density surface, the amount of immobilized SCWP corresponded to 60 resonance units. Samples of rSbsC₃₁₋₂₇₀ and rSbsC₃₁₋₄₄₃ representing the soluble analytes were freshly

TABLE 3. K_d values for the C-terminally truncated rSbsC forms

S-layer protein form	K_d for binding site with indicated affinity:		
	High	Medium	Low
rSbsC ₃₁₋₂₇₀	1.94×10^{-12}	4.80×10^{-9}	9.32×10^{-5}
rSbsC ₃₁₋₄₄₃	2.05×10^{-12}	1.22×10^{-8}	2.95×10^{-6}

prepared as described in reference 15. Solutions of rSbsC₃₁₋₂₇₀ and rSbsC₃₁₋₄₄₃ at concentrations of 5 nM, 2.5 nM, 1.25 nM, 625 pM, 313 pM, 156 pM, 78 pM, 39 pM, 19.5 pM, 9.75 pM, and 4.88 pM and the buffer solution (50 mM Tris-HCl buffer, pH 7.2) were passed over the SCWP-coated sensor surface, as well as the flow cell with blank immobilization, for 1.5 min. The dissociation of bound proteins in the flow of HEPES-buffered saline was recorded for 2 min. Figure 3 shows the association and dissociation curves determined after the subtraction of the reference surface values for five selected concentrations (78 pM, 39 pM, 19.5 pM, 9.75 pM, and 4.88 pM) of rSbsC₃₁₋₂₇₀ (panel A) and rSbsC₃₁₋₄₄₃ (panel B) passed over a flow cell coated with the SCWP of *G. stearotheophilus* ATCC 12980. The measured data were interpreted in terms of three kinds of binding sites for the N-terminal region of the S-layer protein rSbsC according to a model modified by Mader et al. (15) in order to handle three parallel reactions. Data evaluation according to this model allowed a fairly good fit and an interpretation of the binding behavior of rSbsC₃₁₋₂₇₀ and rSbsC₃₁₋₄₄₃ exhibiting low, medium, and high affinities, as presented in Table 3. In contrast, neither rSbsC₄₄₃₋₁₀₉₉ nor rSbsC₆₃₈₋₁₀₉₉ revealed any affinity for the SCWP of *G. stearotheophilus*

FIG. 3. Response curves recorded for the binding of rSbsC₃₁₋₂₇₀ (A) and rSbsC₃₁₋₄₄₃ (B) at concentrations of 78 pM (lines a), 39 pM (lines b), 19.5 pM (lines c), 9.75 pM (lines d), and 4.88 pM (lines e) to the SCWP of *G. stearotheophilus* ATCC 12980 immobilized on a CM5 chip. Overlaid sensorgrams in panel C demonstrate the binding of rSbsC₃₁₋₂₇₀ (lines a and c) and rSbsC₃₁₋₄₄₃ (lines b and d) at a concentration of 2.5 nM to the SCWP of *G. stearotheophilus* ATCC 12980 (lines a and b) and the SCWP of *B. sphaericus* CCM 2177 (lines c and d) immobilized on a CM5 chip. The response curves for the binding of the SCWP of *G. stearotheophilus* ATCC 12980 at concentrations of 38 nM (line a), 19 nM (line b), 9.5 nM (line c), 4.75 nM (line d), 2.375 nM (line e), 1.19 nM (line f), 0.6 nM (line g), and 0.3 nM (line h) to immobilized rSbsC₃₁₋₄₄₃ are shown in panel D. All studies were performed at a flow rate of 30 $\mu\text{l min}^{-1}$.

ATCC 12980 (data not shown), confirming that the C-terminal region of the S-layer protein is not involved in the binding to the cell wall. The K_d values were in good agreement with those obtained for the SLH domain-carrying S-layer protein SbsB and the corresponding pyruvylated SCWP, for which three binding sites with low ($K_d = 2.6 \times 10^{-5}$ M), medium ($K_d = 6.1 \times 10^{-8}$ M), and high ($K_d = 6.7 \times 10^{-11}$ M) affinities have been described previously (15). Furthermore, for rSbpA_{31–318}, the minimal SCWP-binding form of the S-layer protein SbpA, three K_d values corresponding to low ($K_d = 2.4 \times 10^{-2}$), medium ($K_d = 3.0 \times 10^{-8}$), and high ($K_d = 1.2 \times 10^{-11}$) affinities could be determined. For all three S-layer proteins, the high-affinity binding site could be explained by avidity effects caused by the binding of at least dimers (15, 34), since it has been reported previously that even in monomeric protein solutions, small amounts of multimeric aggregates cannot be excluded. The existence of two other binding sites with low and medium affinities may be attributed to heterogeneities associated with naturally occurring polysaccharides (15).

For comparative qualitative binding experiments, 2.5 nM rSbsC_{31–270} and rSbsC_{31–443} were passed for 1 min over a sensor surface onto which either the SCWP of *Bacillus sphaericus* CCM 2177 or the SCWP of *G. stearothermophilus* ATCC 12980 was immobilized after the introduction of carbonylhydrazide at the reducing end of the SCWP (15). As indicated by the sensorgram (Fig. 3C), rSbsC_{31–270} and rSbsC_{31–443} showed, as expected, high levels of specific binding to the SCWP of *G. stearothermophilus* ATCC 12980 (Fig. 3C, a and b) but no affinity for the SCWP of *B. sphaericus* CCM 2177 (Fig. 3C, c and d), which is also negatively charged but has a different chemical composition. Therefore, the binding of the positively charged N-terminal part of SbsC to the SCWP by mere electrostatic interaction can be excluded.

Qualitative binding experiments were performed using the inverted setup, in which rSbsC_{31–270} and rSbsC_{31–443} were immobilized on the Biacore CM5 sensor chip. Therefore, the standard amine coupling method was applied using the application wizard for surface preparation according to the manufacturer's protocol (2). To obtain buffer conditions optimized for the coupling of the samples to distinct flow cells, the pH of the 10 mM acetate buffer was adjusted according to the isoelectric points (pIs) of the rSbsC forms (Table 2). Different concentrations (9.5 nM, 4.75 nM, 2.38 nM, 1.19 nM, 0.6 nM, and 0.3 nM) of the SCWP of *G. stearothermophilus* ATCC 12980, dissolved and diluted in running buffer, as well as the buffer solution (50 mM Tris-HCl buffer, pH 7.2), were injected and passed over the sensor surfaces with covalently bound rSbsC_{31–270} (data not shown) or rSbsC_{31–443} (Fig. 3D) for 3 min. The SCWP showed affinity for rSbsC_{31–270}, as well as for rSbsC_{31–443}.

The results from both complementary SPR setups confirmed that the interaction between the mannosamine uronic acid-containing SCWP of *G. stearothermophilus* ATCC 12980 and the N-terminal region comprising amino acid residues 31 to 270 is highly specific and that analogous to the SLH domain, this region constitutes the functional SCWP-binding region. The affinity constants obtained for SbsC and the corresponding SCWP are in the range of those determined for the binding mechanism involving SLH motifs and pyruvylated SCWPs.

In contrast to SbsB, the S-layer protein SbpA of *B. sphaeri-*

cus CCM 2177 requires, in addition to the three SLH motifs, an adjacent 58-amino-acid-long SLH-like motif reaching from amino acids 213 to 270 to reconstitute the functional SCWP-binding domain (8). Interestingly, in SbpA, as well as in SbsC, the functional SCWP-binding domain comprises the N-terminal amino acid residues 31 to 270 and has been found to be organized mainly as α -helices (10, 12, 18).

To conclude, the highly specific lectin-type binding between S-layer proteins and SCWPs is an important mechanism for generating and maintaining a dynamic protein crystal on a bacterial cell surface during all stages of cell growth and division. The SCWP-mediated anchoring of S-layer subunits to the rigid cell wall layer has a high degree of biological relevance since it guarantees a defined orientation and incorporation of the S-layer protein upon reaching the cell surface while allowing enough flexibility for the recrystallization of S-layer subunits to continuously assume a low-free-energy arrangement during cell growth and cell division (27).

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