The S-layer homology domains of *Paenibacillus alvei* surface protein SpaA bind to cell wall polysaccharide through the terminal monosaccharide residue

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Self-assembling (glyco)protein surface layers (S-layers) are ubiquitous prokaryotic cell-surface structures involved in structural maintenance, nutrient diffusion, host adhesion, virulence, and other processes, which makes them appealing targets for therapeutics and biotechnological applications as biosensors or drug delivery systems. However, unlocking this potential requires expanding our understanding of S-layer properties, especially the details of surface-attachment. S-layers of Gram-positive bacteria often are attached through the interaction of S-layer homology (SLH) domain trimers with peptidoglycan-linked secondary cell wall polymers (SCWPs). Cocrystal structures of the SLH domain trimer from the *Paenibacillus alvei* S-layer protein SpaA (SpaASLH) with synthetic, terminal SCWP disaccharide and trisaccharide analogs, together with isothermal titration calorimetry binding analyses, reveal that while SpaASLH accommodates longer biologically relevant SCWP ligands within both its primary (G2) and secondary (G1) binding sites, the terminal pyruvylated ManNAc moiety serves as the nearly exclusive SCWP anchoring point. Binding is accompanied by displacement of a flexible loop adjacent to the receptor site that enhances the complementarity between protein and ligand, including electrostatic complementarity with the terminal pyruvate moiety. Remarkably, binding of the pyruvylated monosaccharide SCWP fragment alone is sufficient to cause rearrangement of the receptor-binding sites in a manner necessary to accommodate longer SCWP fragments. The observation of multiple conformations in longer oligosaccharides bound to the protein, together with the demonstrated functionality of two of the three SCWP receptor-binding sites, reveals how the SpaASLH–SCWP interaction has evolved to accommodate longer SCWP ligands and alleviate the strain inherent to bacterial S-layer adhesion during growth and division.

Prokaryotic surface layers (S-layers) are composed of (glyco)protein subunits that self-assemble into two-dimensional paracrystalline lattices attached to the exterior of the cell envelope (Fig. 1) (1–7). As one of the most abundant prokaryotic protein systems, S-layer functions are varied and involve biological processes such as cell shaping, structural maintenance, nutrient diffusion, host adhesion, and virulence, among others (4, 8–11), and provide an excellent opportunity for studying fundamentals of protein transport, structure, and assembly. Together with their high surface accessibility, these features make S-layers appealing therapeutic targets with significant biotechnological potential as both biosensors and as drug delivery systems (4, 12–19). Expanding our understanding of S-layer surface-attachment represents an essential step toward exploiting this potential.

To maintain S-layer function while coping with the structural demands of cellular growth and division (4), prokaryotes have evolved covalent and noncovalent S-layer cell-matrix anchoring mechanisms that rely on high affinity but nevertheless flexible attachments to cell wall structures. While S-layers are found in archaea (4, 20, 21) and Gram-negative bacteria (9, 22, 23), only in Gram-positive bacteria are they anchored to the cell wall via evolutionarily conserved S-layer homology (SLH) domains (4, 9, 24–26), which generally occur as triplicate repeats (SLH1, SLH2, and SLH3) located at the protein C-terminus or immediately after the N-terminal signal peptide (4, 9, 24–26). SLH domains have been shown to bind noncovalently neutral or anionic polysaccharides called non-classical secondary cell wall polymers (SCWPs) that are themselves covalently linked to the muramic acid residues of peptidoglycan (Fig. 1) (11).

Recently published structures of the SLH-domain trimers from the S-layer proteins SpaA (*Paenibacillus alvei*) (27) and Sap (*Bacillus anthracis*) (28, 29) show overall threefold symmetry, where the ~55 residue repeats each consist of two α-helices connected by an extended loop (27–29). The C-terminal helix from each SLH repeat contributes to a three-helix core bundle parallel to the threefold symmetry axis, while the corresponding N-terminal helix is rotated ~90° from the threefold axis to form three alternating lobes and grooves (27, 28). The N-terminus of each core helix for *P. alvei* SpaA contributes a conserved “TRAE” motif toward these binding grooves, where TRAE of SLH1, TVEE of SLH2, and TRAQ of

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SLH3 are primarily situated within grooves 1 (G1), 2 (G2), and 3 (G3), respectively (27, 28). The side chains of the arginine residues of the TRAE (SLH1) and TRAQ (SLH3) motif extend through the hydrophobic core to emerge at the base of the neighboring grooves G2 and G1, respectively, where their positively charged guanidino groups are positioned to complement the negatively charged SCWP ligands.

*P. alvei* CC M 2051 T (henceforth *P. alvei*) SCWP is composed of 11→3)-4,6-Pyr-β-D-ManpNAc-(1→4)-β-D-GlcpNAc-(1→ repeats (30). The binding of a synthetic, terminal 4,6-Pyr-β-D-ManpNAcOMe SCWP monosaccharide analog to an SLH domain has been shown to induce a remarkable structural rearrangement involving an ~180° phi angle backbone flip of a conserved glycine residue (consensus sequence SLH-Gly29) fundamental to the receptor site’s ability to bind ligand, including accommodation of the negative charge of the terminal pyruvate moiety (27). Together with enzymatic and biochemical analyses, these structural findings confirm that, for SpaA, Sap, and other Gram-positive S-layer proteins, SLH-mediated SCWP anchoring is reliant on a negatively charged ketal-pyruvate moiety located on the terminal sugar residue of the SCWP (27, 29–33).

*P. alvei* SpaASLH monosaccharide-bound structures identified separate SLH-domain binding sites in G2 and G1 (27). Specifically, wild-type SpaASLH protein was first observed with ligand bound in G2, and upon inactivation of binding in G2 through a mutation of its conserved glycine residue to alanine (G109A), a remarkable conformational shift was observed where the mutant construct instead utilized G1 for binding with a concomitant tenfold decrease of the associated ITC monosaccharide binding affinity (27). The double mutation of conserved glycine residues in G1 and G2 to alanine (G46A/G109A) abolished SpaASLH’s ligand-binding ability (27). Together, these observations show that monosaccharide SCWP binding is likely centered about G2, with G1 serving as the alternate site for SCWP binding (27).

Figure 1. The cell surface of many prokaryotes is coated in a surface layer (S-layer) consisting of one or more (glyco)proteins arranged in a repeating two-dimensional array. A, schematic diagram of S-layer protein binding on Gram-positive bacteria mediated via specialized S-layer homology (SLH) domains that recognize and bind peptidoglycan-linked secondary cell wall polymers (SCWPs), with the inset showing the structure of the SCWP from *P. alvei* CC M 2051 T (11, 27, 30); and B the synthetic ligand analogs used in this study corresponding to the terminal disaccharide and trisaccharide units of the *P. alvei* CC M 2051 T SCWP ligand.
Significantly, the ability of SpaASLH to accommodate monosaccharide binding in G2 or G1 suggests a biological mechanism for the relief of S-layer strain encountered during processes of cellular growth and division (27), where the transfer of SCWP interactions between SLH-domain binding sites could facilitate S-layer rearrangement and expansion without disrupting the overall confluence of the S-layer protein lattice. To date, however, the analysis of this novel binding site switching mechanism has been limited to the terminal monosaccharide 4,6-Pyr-β-D-ManNAcOMe and the internal repeat disaccharide β-D-GlcNAc-(1→3)-4,6-Pyr-β-D-ManNAcOMe SCWP ligand analogs (27), leaving open the role of the subsequent SCWP sugar residues in binding, as well as whether the primary G2 and secondary G1 sites can accommodate longer ligands that better approximate SCWP at the surface of P. alvei. To address this, here, we characterize the details of the SpaASLH-SCWP interaction using thermodynamic isothermal titration calorimetry (ITC) analysis of disaccharide and trisaccharide binding and through the determination of the cocrystal structures of SpaASLH and single (SpaASLH/G109A) and the corresponding double (SpaASLH/G46A/G109A) mutants in complex with synthetic terminal disaccharide and trisaccharide analogs of the P. alvei SCWP ligand.

Results

Binding parameters of SpaASLH and disaccharide and trisaccharide SCWP analog

ITC was used to assess the ability of SpaASLH, SpaASLH/G109A, and SpaASLH/G46A/G109A to bind the terminal 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAcOMe disaccharide analog and the 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAc-(1→3)-4,6-Pyr-β-D-ManNAcOMe trisaccharide analog in solution (Table 1). The disaccharide binds with 1:1 stoichiometry and an apparent dissociation constant ($K_D$) of 64 nM at 20 °C for the wild-type protein SpaASLH and of 288 nM at 20 °C for the SpaASLH/G109A mutant (Table 1). The binding affinity toward the disaccharide was determined to be slightly lower compared with the monosaccharide (27) (Table 1) while interestingly, the trisaccharide analog shows a slightly lower $K_D$ (4.7 nM at 20 °C) implicating a slightly higher binding affinity toward the wild-type protein SpaASLH.

With the G109A single mutant, the $K_D$ drops 55-fold (260 nM at 20 °C) compared with trisaccharide binding data for the wild-type protein (27). Despite a similar Gibbs free energy ($\Delta G$), the entropic contribution is comparably stronger with both trisaccharide and disaccharide compared with the monosaccharide (Table 1) resulting in an overall lower enthalpic contribution. For visualization, a comparison of the binding energies is shown in Fig. S1; and binding isotherms are shown in Fig. S2. The binding data of the di- and trisaccharide support a favorable hydrogen bond formation and hydrophobic interaction, while the binding data of the monosaccharide (27) support a good hydrogen bonding with unfavorable conformational change. With neither of the synthetic SCWP analogs, binding is observed with the SpaASLH/G46A/G109A double mutant (Table 1).

Table 1

| Ligand bound to SpaASLH variant | Temperature (°C) | −$\Delta$S (kJ/mol) | ΔH (kJ/mol) | ΔG (kJ/mol) | Stoichiometry | $K_A$ (M$^{-1}$) | $K_D$ (nM) |
|--------------------------------|----------------|---------------------|-------------|-------------|--------------|----------------|-------------|
| Trisaccharide-binding data     |                |                     |             |             |              |                |             |
| SpaASLH                       | 20             | −18.70 ± 5.57       | −27.14 ± 3.29 | −45.84 ± 2.29 | 1.16 ± 0.10  | 2.14 × 10$^2$ ± 1.46 | 4.7         |
| SpaASLH/G109A                 | 20             | −15.99 ± 1.00       | −20.92 ± 0.57 | −36.91 ± 0.47 | 1.44 ± 0.25  | 3.84 × 10$^2$ ± 0.68 | 260        |
| SpaASLH/G46A/G109A            | 20             | No binding          |             |             |              |                |             |
| Disaccharide-binding data      |                |                     |             |             |              |                |             |
| SpaASLH                       | 20             | −9.95 ± 2.08        | −30.36 ± 1.64 | −40.31 ± 0.65 | 1.20 ± 0.16  | 1.56 × 10$^2$ ± 0.37 | 64         |
| SpaASLH/G109A                 | 20             | −15.74 ± 1.35       | −20.95 ± 1.51 | −36.70 ± 0.20 | 1.30 ± 0.19  | 3.47 × 10$^2$ ± 0.29 | 288        |
| SpaASLH/G46A/G109A            | 20             | No binding          |             |             |              |                |             |
| Previously published (27)      |                |                     |             |             |              |                |             |
| monosaccharide-binding data    |                |                     |             |             |              |                |             |
| SpaASLH                       | 20             | 45.74 ± 16.01       | −87.85 ± 15.62 | −42.10 ± 0.65 | 0.91 ± 0.04  | 3.48 × 10$^2$ ± 0.36 | 29         |
| SpaASLH/G109A                 | 20             | 13.33 ± 7.0         | −50.64 ± 7.29 | −37.31 ± 0.33 | 0.92 ± 0.04  | 4.48 × 10$^2$ ± 0.62 | 226        |
| SpaASLH/G46A/G109A            | 20             | No binding          |             |             |              |                |             |

For comparison, the published ITC data for 4,6-Pyr-β-D-ManNAcOMe monosaccharide binding (27) is included.
SpaSLH recognition of pyruvylated terminal SCWP units

Table 2

| Protein | SpaSLH recognition of pyruvylated terminal SCWP units |
|---------|-----------------------------------------------------|
| Ligand  | SpaSLH | SpaSLH | SpaSLH/G109A | SpaSLH/G46A/G109A |
| PDB code | 7SV3 | 7SV4 | 7SV5 | 7SV6 |
| Data collection | | | | |
| Space group | P2₁2₁2₁ | P2₁2₁2₁ | C2 | P2₁2₁2₁ |
| Resolution (Å) | 20.00–1.70 (1.76–1.70) | 20.00–2.06 (2.13–2.06) | 20.00–1.72 (1.78–1.72) | 20.00–1.85 (1.92–1.85) |
| Cell dimensions | a (Å) | b (Å) | c (Å) | a (Å) | b (Å) | c (Å) | a (Å) | b (Å) | c (Å) |
| Z | 4 | 4 | 8 | 4 | 4 | 8 | 4 | 4 | 8 |
| R (Å) | 0.145 (0.773) | 0.143 (0.591) | 0.071 (0.539) | 0.099 (0.540) |
| R (Å) | 0.048 (0.369) | 0.043 (0.295) | 0.029 (0.301) | 0.053 (0.345) |
| Completeness (%) | 100.0 (99.9) | 98.4 (90.1) | 90.3 (82.6) | 98.2 (99.3) |
| Redundancy | 1.70 (1.76) | 1.72 (1.78) | 1.85 (1.92) | 2.06 (2.13) |
| Unique reflections | 17.1 | 17.1 | 17.1 | 17.2 |
| Refinement | | | | |
| Resolution (Å) | 20.00–1.70 | 20.00–2.06 | 20.00–1.72 | 20.00–1.85 |
| No. reflections | 10,409 | 20,968 | 30,968 | 13,083 |
| R (Å) | 18.4 | 18.4 | 18.4 | 18.4 |
| R (Å) | 24.0 | 24.0 | 24.0 | 24.0 |
| No. atoms | 1311 | 1290 | 2520 | 1281 |
| Protein | 1311 | 1290 | 2520 | 1281 |
| Ligand(s) | 35² | 66² | 82² | 35² |
| Water | 242 | 137 | 303 | 112 |
| B factors (Å²) | | | | |
| Protein | 15.1 | 25.0 | 21.2 | 22.4 |
| Ligand | 18.0 | 34.6 | 19.5 | 37.1 |
| Water | 27.5 | 32.6 | 31.6 | 31.6 |
| Average | 17.1 | 26.2 | 22.2 | 23.5 |
| Ramachandran distribution | | | | |
| Favored (%) | 98.2 | 98.8 | 98.5 | 98.8 |
| Allowed (%) | 1.8 | 1.2 | 1.5 | 1.2 |
| Disallowed (%) | 0 | 0 | 0 | 0 |
| r.m.s. bonds (Å) | 0.0039 | 0.0031 | 0.0034 | 0.0040 |
| r.m.s. angles (°) | 1.25 | 1.23 | 1.21 | 1.23 |

¹ Disaccharide = 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAcOMe.
² Trisaccharide = 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAc-(1→3)-4,6-Pyr-β-D-ManNAcOMe.
³ Glycerol = C₃H₈O₃.

196–197) structures, each, display partial main chain and side-chain electron density, which is consistent with previous SpaSLH structures (27). Further, the di- and trisaccharide analogs bound by wild-type SpaSLH (Fig. 2, A and B), as well as the disaccharide analog bound by the SpaSLH/G109A single mutant (Fig. 2C) and SpaSLH/G46A/G109A double mutant (Fig. 2D) all display unambiguous electron density for the entire ligand.

Cocrystallization with SCWP terminal disaccharide and trisaccharide

To investigate the molecular details of SpaSLH SCWP binding, the synthesized P. alvei SCWP terminal disaccharide analog 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAcOMe (35) (Fig. 1B) was cocrystallized with wild-type SpaSLH. The structure reveals unambiguous electron density for the entire disaccharide bound in G2 (Fig. 2A), where the terminal ManNAc residue binds in the same manner to that of the published structure of monosaccharide-bound SpaSLH (27) within a deep, narrow positively charged pocket lined by residues contributed by all three SLH domains. The pyruvate moiety forms a salt-bridge interaction with TRAE residue Arg61 from SLH1 (corresponding to SLH-Arg43) as well as a hydrogen bond to the backbone amide of Gly109 from SLH2 (corresponding to SLH-Gly29), while the terminal ManNAc ring forms a stacking interaction with Trp151 from SLH3 (corresponding to SLH-Trp13) (Fig. 2A). The disaccharide β(1→4) linkage extends past the edges of the binding pocket, which forces the GlcNAc residue to project out from the binding site without forming direct hydrogen bond contacts with SpaSLH surface residues, although it does interact through an ordered network of bridging water molecules to surface residues Lys110, Glu127, and Trp151 (Fig. 3A).

The P. alvei SCWP terminal trisaccharide analog 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAc-(1→3)-4,6-Pyr-β-D-ManNAcOMe (Fig. 1B) was also synthesized (see Supporting information) and cocrystallized with wild-type SpaSLH. The resultant cocrystal structure reveals clear electron density for the entire trisaccharide ligand bound to the G2 pocket (Fig. 2B). However, while the terminal, pyruvyalted ManNAc residue binds in the same manner as observed for the disaccharide (above) and monosaccharide (27) SpaSLH structures (Fig. 3B), the GlcNAc residue adopts a rotated conformation...
where the acetamido moiety is displaced 4.5 Å toward the opposite face of the binding pocket, to form a single hydrogen bond contact with the protein surface and bridging water molecule contacts with Lys110, Glu127, and Thr149 (Fig. 3B). This results in the GlcNAc O3 hydroxyl being shifted by 2.1 Å with respect to the disaccharide, to form a hydrogen bond and water bridge to Trp151 and Glu127, respectively (Fig. 3B). In trisaccharide-bound SpaASLH, the internal-repeat ManNAc residue forms a 3.2 Å hydrogen bond to the GlcNAc acetamido moiety and bridging water interactions to surface residues Lys110 and Asp150 (Fig. 3B).

The terminal disaccharide analog also cocrystallized in G2 of SpaASLH/G46A/G109A, with the overall protein structure exhibiting a high degree of structural homology to disaccharide-bound SpaASLH (with an overlap Ca rmsd of 0.44 Å), while displaying unambiguous electron density for the entire ligand (Fig. 2D).

The terminal disaccharide analog was also cocrystallized with the SpaASLH/G109A single mutant designed to destabilize ligand binding in G2. The structure displays unambiguous electron density for the entire disaccharide ligand within G2 (Fig. 2C), where the terminal ManNAc ring of the disaccharide sits deep within the G1 pocket lined by conserved residues Arg177 of SLH3 (corresponding to conserved residue SLH-Arg43), Trp93 of SLH2 (corresponding to the conserved residue SLH-Trp13), and Gly46 (corresponding to conserved residue SLH-Gly29). The disaccharide’s β(1→4) linkage does not extend entirely past the mouth of the G1 pocket, and the lower portion of the GlcNAc sugar residue remains partially obscured by residues Pro48 and Trp93 of SLH2 (Fig. 3C). The extended GlcNAc residue does not form any direct hydrogen bonds with the protein, but derives partial stability from hydrophobic contacts with Pro48 and Trp93 (Fig. 3C). The GlcNAc O3 hydroxyl simultaneously forms a water-bridge with Glu63, while coordinating the ManNAc O5 hydroxyl and GlcNAc N-acetyl nitrogen (Fig. 3C). The GlcNAc acetamido substituent forms a hydrogen bond with a surface-associated glycerol molecule (Fig. 3C).

**GlcNAc sugar occupancy in disaccharide-bound SpaASLH**

In the cocrystal structure of disaccharide-bound SpaASLH, unexplained electron density was observed in G2 near the GlcNAc sugar moiety of the ligand, which could be explained by the GlcNAc sugar residue being disordered over two conformations (Fig. 3D), with the second conformation (Conformation 2) lying in the difference density. The occupancy of the primary GlcNAc conformation (Conformation 1) was adjusted to 50% to yield temperature factors comparable with those of surrounding protein and ligand atoms, ranging between 10.00 and 19.00.

In conformation 1, the acetamido and O3 hydroxyl groups are positioned nearest the region of the binding pocket where residue Lys110 is located (Fig. 3A). In conformation 2, the

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Figure 2. Ribbon models for each SpaASLH co-crystal structure in Table 2. 2Fo-Fc electron density maps contoured to 1σ shown for the corresponding model of bound ligand for (A) disaccharide-bound SpaASLH; (B) trisaccharide-bound SpaASLH; (C) disaccharide-bound SpaASLH/G109A with ligands shown from both molecules of the asymmetric unit of the co-crystal structure; and (D) disaccharide-bound SpaASLH/G46A/G109A double mutant. Grooves 1, 2, and 3 are indicated on each structure as G1, G2, and G3, respectively. SLH1 orange, SLH2 blue, and SLH3 aquamarine. Ligand atoms are colored by element with oxygen red, nitrogen blue, and carbon white. 2Fo-Fc electron density maps are depicted as gray mesh.
acetamido and O3 hydroxyl groups are displaced by 4.5 Å and 2.1 Å, respectively, approaching the opposite side of the binding pocket where residues Trp151 and Thr149 are located, and adopting a conformation close to that of the terminal ManNAc and GlcNAc residues from the trisaccharide ligand bound to SpaASLH (Fig. 3B).

Discussion

The crystal structures of wild-type and mutant SLH trimers from the *P. alvei* S-layer protein SpaA (SpaASLH) determined in the presence of the SCWP terminal monosaccharide analog reported earlier (27) demonstrated the remarkable conformational flexibility of the trimer while switching between the G2 and G1 SCWP binding sites and suggested a mechanism for relieving the strain encountered by S-layers during processes of cellular growth and division. The biological relevance of the binding site switch was demonstrated in that report (27) using synthetic, terminal pyruvylated ManNAc monosaccharide. To explore this observation, we now report the crystal structures and binding characteristics of wild-type and mutant SpaASLH determined in the presence of the SCWP terminal di- and trisaccharide analogs (Fig. 1B).

Interestingly, compared with the nanomolar binding that wild-type SpaASLH achieved for the terminal SCWP monosaccharide (27), it showed now a slight decrease in binding affinity for the SCWP terminal disaccharide and a slight increase for the trisaccharide. Di- and trisaccharide analogs were observed to cocrystallize in G2 of wild-type SpaASLH (Fig. 2A and B), while disaccharide was observed to cocrystallize in G1 of the conformationally rearranged SpaASLH/G109A single mutant (Fig. 2C) designed to destabilize binding in G2 (Table 1) and, again surprisingly, in G2 of the SpaASLH/G46A/G109A double mutant (Fig. 2D).

**G2 of SpaASLH accommodates ligand flexibility at the penultimate SCWP sugar residue**

In both the di- and trisaccharide-bound wild-type SpaASLH co-crystal structures, the terminal monosaccharide residue...
forms contacts to the G2 site, with the remaining oligosaccharide residues extending into solvent where they form bridging contacts to the protein surface through a network of ordered water molecules (Fig. 3, A and B). Interestingly, the disaccharide bound to SpaASLH is observed to adopt two distinct conformations (Conformations 1 and 2, Fig. 3D), while the bound trisaccharide is observed in a single conformation (Fig. 3B) corresponding to conformation 2 of the disaccharide, allowing the GlcNAc O3 to form a hydrogen bond with the Trp151 side chain nitrogen while the internal repeat ManNAc acetamido forms a 3.2 Å hydrogen bond (Fig. 3B) to the nitrogen atom of the GlcNAc acetamido. These conformational differences alone demonstrate that G2 accommodates significant flexibility in SCWP ligands by enabling the SCWP to pivot about the anchored terminal ManNAc.

It is interesting to compare the structure of SpaASLH bound to the SCWP terminal disaccharide (4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAcOMe) with the structure published from our group of SpaASLH bound to internal disaccharide (β-D-GlcNAc-(1→3)-4,6-Pyr-β-D-ManNAcOMe) (27), where the internal ManNAc residue is seen bound to the protein in largely the same manner as the terminal ManNAc residue. The lack of corresponding measurable binding in ITC experiments with the internal-repeat SCWP (27) indicates that this phenomenon is unlikely to represent a common biological interaction, but it does emphasize the flexibility of G2 as an adaptable primary SCWP-binding site.

### Disaccharide in G1 of SpaASLH/G109A lies in an intermediate conformation

The deactivation of G2 using the G109A mutation forces SpaASLH to utilize G1, which binds the longer, more biologically relevant SCWP terminal disaccharide ligand in a conformation intermediate between conformations 1 and 2 observed in G2 of wild-type SpaASLH. As observed for binding of the disaccharide in G2 of the SpaASLH wild-type, only the terminal monosaccharide residue contacts the G1-binding site of the SpaASLH/G109A single-mutant (Fig. 1D), while the GlcNAc residue extends into solvent to form bridging contacts with the protein surface via an ordered network of water molecules (Fig. 3C).

### Binding of the terminal SCWP monosaccharide in itself causes displacement of residues outside the monosaccharide receptor site required to accommodate longer SCWP oligosaccharides

Superposition of the monosaccharide receptor sites in the unliganded wild-type SpaASLH and the corresponding disaccharide-bound forms shows that the binding of SCWP fragments longer than a monosaccharide in G2 in wild-type SpaASLH and in G1 in SpaASLH/G109A requires displacement of a number of amino acid residues to avoid steric collision with internal saccharide residues. However, in a fascinating observation, the same relative displacements (27) are observed in the published structures of both proteins bound to monosaccharide. That is, the binding of the monosaccharide alone is sufficient to cause rearrangement of the receptor-binding sites necessary to accommodate longer SCWP fragments.

In disaccharide-bound wild-type SpaASLH, the α-carbon atoms of residues SLH-30-31-32 (Lys110-Asp111-Leu112) that border the G2 monosaccharide receptor site and are adjacent to conserved residue SLH-Gly29 (Gly-109) are displaced by 1.0 Å, 1.1 Å, and 1.4 Å, respectively, relative to the SLH-30-31-32 α-carbon atoms in the unliganded structure (Fig. 4A). The monosaccharide acetamide moiety triggers this conformational shift first by displacing Leu112 away from the opening of G2. Together with the conserved Gly109 backbone flip, the Lys110 main chain atoms are forced toward the receptor site and stretch the Lys110 side chain atoms into near-full extension, which can accommodate the SCWP disaccharide GlcNAc residue (Fig. 4B).

A corresponding and somewhat more dramatic rearrangement occurs in G1 of the single mutant (Fig. 4C) through a slightly different mechanism. In the monosaccharide-bound G109A mutant, after compensating for the rearrangement that occurs during the conformational shift, the α-carbon atoms of G1 residues SLH-30-31-32 (Tyr47-Pro48-Gly49) are displaced by 4.6 Å, 6.4 Å, and 5.2 Å, respectively, relative to the unliganded structure (Fig. 4C). Specifically, monosaccharide binding directly displaces Pro48 as it stacks against the acetamide moiety, clearing the way for internal oligosaccharide residues (Fig. 4D) while concomitantly moving Tyr47 and Gly49. Similar changes are seen in the disaccharide-bound G109A mutant, where the rearrangements in G1 accommodate the corresponding terminal pyruvylated ManNAc residue and subsequent GlcNAc residue (Fig. 4D).

### Binding affinity for G1 in SpaASLH/G109A is significantly weaker compared with G2 in the wild-type SpaASLH

SpaASLH has three potential receptor sites, which complicates binding affinity determinations. Binding assays carried out by mutating the three SLH domain motifs TRAE (SLH1), TVEE (SLH2), and TRAQ (SLH3) respectively to TAAA reduced binding to 37%, 88%, and 50%, respectively (36, 37) relative to wild-type. Co-crystallization of wild-type SpaASLH with a molar excess of SCWP fragments showed significant occupation of only the G2 site (27). Blackler et al. (27) explored the role of conserved residue SLH-Gly29 through sequential mutation to alanine and showed that by deactivating the G2 site through a G109A mutation, the protein underwent a conformational shift that enabled G1 to bind both the monosaccharide and internal disaccharide SCWP fragments, though with a significant reduction in affinity compared with wild-type (Table 1). When bound, G1 and G2 each make important contacts with the terminal, pyruvylated SCWP monosaccharide through residues SLH-Trp13, SLH-Gly29, and SLH-Arg43. Yet the approximate geometries of the binding sites differ, including at residue positions SLH 30-31-32.

In unliganded SpaASLH, G1 adopts a slightly more “open” conformation (measuring 9.1 Å between Trp93 Cβ2 and Gly46 O) than G2 (measuring 8.6 Å between Trp151 Cβ2 and Gly109
O). Ligand binding in either of G1 or G2 reduces the width of the receptor site to \( \frac{\Delta r}{R} = 7.5 \) Å, which means that G1—initially in a more open conformation prior to ligand binding—undergoes an additional 0.5 Å displacement to accommodate the SCWP interaction to adopt a geometry similar to that of G2-liganded SpaASLH. The binding of ligand in G2 does not significantly affect the open conformation of G1 observed in the completely unliganded state.

The conformational change observed in the G109A mutant features displacement of the SLH2 domain toward SLH1 (27), as well as a \( > 4.5 \) Å shift in G1 residues SLH 30-31-32 (Fig. 4, C and D). The latter SLH 30-31-32 movement occurs perpendicular to the SLH2 domain shift. The net result is a liganded G1 site with a strong resemblance to that of G2 (Fig. 4).

Collectively, the larger groove-narrowing and polypeptide loop shifts of G1 are consistent with the observed reduction in SpaASLH/G109A binding affinity ((27) and Table 1) relative to SpaASLH wild-type.

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Figure 4. SpaASLH residues SLH 30-31-32 that border the monosaccharide receptor site shift in response to terminal ManNAc binding to accommodate longer, more biologically relevant SCWP ligands. A, G2 of unliganded SpaASLH (purple; PDB 6CWH) showing the relative displacement of these residues in G2 caused by binding of monosaccharide. B, the overlap of G2 of disaccharide-bound SpaASLH (green) with G2 of monosaccharide-bound SpaASLH (purple; PDB 6CWH) shows similar shifts, indicating that it is the binding of the monosaccharide, which is largely responsible for the shifts. C, G1 of unliganded SpaASLH (grey) is overlapped with G1 of monosaccharide-bound SpaASLH/G109A single mutant (purple; PDB 6CWN) showing the relative displacement of these residues in G1 caused by binding of the monosaccharide. D, the overlap of G1 of disaccharide-bound SpaASLH/G109A (green) with G1 of monosaccharide-bound SpaASLH (purple, PDB: 6CWN) shows similar shifts, indicating again that it is the binding of the monosaccharide, which is largely responsible for the shifts. All carbon atoms are colored according to the model descriptions above, with oxygen and nitrogen atoms colored in red and blue, respectively.

Doubly deactivated SpaASLH/G46A/G109A mutant cocrystal structure accepts oligosaccharide ligand in G2

The introduction of a single G109A mutation in the G2 binding site of SpaASLH was sufficient to shift all observed binding to the heretofore unoccupied G1 site. The simultaneous deactivation of both G1 and G2 by glycine to alanine mutations results in no observed binding by ITC (Table 1); however, consistent with the monosaccharide-bound SpaASLH/G46A/G109A structure, where binding in the crystalline environment is significantly enhanced compared with in solution (27), the crystal structure of the double mutant cocrystallized with approximate fivefold molar excess of disaccharide does show electron density in G2 corresponding to a fully occupied disaccharide SCWP fragment in Conformation 1 (Fig. 2D). In the presence of ligand in the crystal structure, the double mutant residues SLH 30-31-32 still undergo some displacement relative to the unliganded wild-type SpaASLH, even though the Gly109Ala deactivating mutation...
prevents the G2 backbone flip, indicating the importance of the flip in the recognition and binding of the terminal monosaccharide.

**SCWP binding in the SLH domains of Bacillus anthracis induces residue shifts similar to those observed in SpaASLH**

Comparison of the published liganded (PDB: 6BT4 (29)) and unliganded (PDB: 3PYW (28)) B. anthracis SapSLH structures reveals that G2 residues SHL 30-31-32 (Thr90-Gly91-Asn92) shift upon binding in a manner similar to that observed for P. alvei SpaASLH. This displacement is necessary to prevent steric collision with the bound oligosaccharide SCWP ligand. The SapSLH binding site bears a strong structural similarity to that of SpaASLH, and the shifting SapSLH residues, Thr90-Gly91-Asn92, are proximal to conserved residue SLH-Gly29. Both of these observations suggest that the triresidue shift, Gly91-Asn92, are proximal to conserved residue SLH-Gly29.

The SpaASLH protein, consisting of amino acids 21 to 193 with a Ser-Gly-Ser linker followed by a C-terminal His6 tag, and its single mutant (G109A) and double mutant (G46A/G109A) variants were produced and purified as described previously (27). Overexpression took place at 37 °C with 200 rpm shaking and was induced via addition of IPTG to a final concentration of 0.6 mM once cells reached an OD600 of ~0.6. Following induction, cells were incubated for a further 3 h before harvest by centrifugation at 6500 rpm for 25 min. Cell pellets were resuspended in lysis buffer and lysed by sonication. The lysate was centrifuged at 10,000 g for 20% glycerol for 10 to 20 s prior to being frozen at 100 °C.

**Experimental procedures**

**SpaASLH production and purification**

The SpaASLH protein, consisting of amino acids 21 to 193 with a Ser-Gly-Ser linker followed by a C-terminal His6 tag, and its single mutant (G109A) and double mutant (G46A/G109A) variants were produced and purified as described previously (27). Overexpression took place at 37 °C with 200 rpm shaking and was induced via addition of IPTG to a final concentration of 0.6 mM once cells reached an OD600 of 0.6. Following induction, cells were incubated for a further 3 h before harvest by centrifugation at 6500 g for 25 min. Cell pellets were resuspended in lysis buffer and lysed by sonication. The lysate was centrifuged at 10,000 g, and the protein was purified from the supernatant using a combination of nickel NTA-affinity chromatography (Qiagen) and size exclusion chromatography (SEC), with a yield of ~25 mg of each protein per liter of culture and a purity of >95% based on SDS-PAGE analysis of the SEC fractions (27).

**SpaASLH isothermal titration calorimetry**

ITC experiments were performed as described in (27) with minor modifications. The concentrations of the disaccharide and trisaccharide ligand are given by weight per volume (w/v). Protein concentrations were measured spectrophotometrically at 280 nm using a molar extinction coefficient of 18,450 M⁻¹ cm⁻¹. For the ITC measurements with the disaccharide, a concentration of 500 μM was used for titration against SpaASLH and SpaASLH/G109A at a concentration of 12.5 μM (protein-to-ligand ratio of 1:40). The trisaccharide was titrated at a concentration of 340 μM against an 8.5-μM solution of SpaASLH and at a concentration of 400 μM against a 10-μM solution of SpaASLH/G109A, respectively (protein-to-ligand ratio of 1:40). For the double mutant SpaASLH/G46A/G109A, a ratio of 1:50 was used (500 μM disaccharide to 10-μM solution of SpaASLH/G46A/G109A and 1000 μM trisaccharide to 20-μM solution of SpaASLH/G46A/G109A). The experiments were performed at least in triplicates.

**SpaASLH crystallization**

Recombinant, purified wild-type SpaASLH, SpaASLH/G109A, and SpaASLH/G46A/G109A were concentrated using 3-kDa MWCO Amicon ultra centrifugal filter units and were cocrystallized with either 5 mM 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAcOMe disaccharide analog (35) or 1.1 mM 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAc-(1→3)-4,6-Pyr-β-D-ManNAcOMe trisaccharide analog (for the synthesis, see Supporting information; Figs. S3–S5; Schemes S1–S3). Screens were prepared using an Art Robbins Instruments crystal gryphon robot and Hampton 96-well Intelli plates. Cocrystals of SpaASLH (15 mg/ml) and disaccharide were obtained within 1 week from Hampton Index screen condition number 2 (0.1 M sodium acetate trihydrate pH 4.5, 2.0 M ammonium sulphate). Cocrystals of SpaASLH/G109A (12 mg/ml) and disaccharide were obtained after 1 month from the Qiagen PEG II screen condition 15 (0.1 M HEPES pH 7.5, 25% (w/v) PEG 1000). Cocrystals of SpaASLH/G46A/G109A (19 mg/ml) and disaccharide were obtained after 1 week from the Qiagen PEG II screen condition 68 (0.1 M sodium acetate, 25% (w/v) PEG 4000, 8% (w/v) isopropanol). Cocrystals of SpaASLH (18 mg/ml) and trisaccharide were obtained within 1 week from Qiagen JCSG+ screen condition number 83 (0.2 M Magnesium Chloride, 0.1 M Bis-Tris pH 5.5, 25% PEG 3350).

**Data collection, structure solution, and refinement**

Each crystal was resuspended in mother liquor containing 20% glycerol for 10 to 20 s prior to being flash-frozen at 100 K for data collection. For all structures, X-ray diffraction data were collected using a Dectris Pilatus 200 K detector coupled to a Rigaku Micromax-007 HF X-ray generator, and the data were scaled, averaged, and integrated using HKL2000. To generate a structure solution, molecular replacement was performed using Phaser from the CCP4 suite (34), with unliganded SpaASLH (PDB: 6CWM) used as a search model for the SpaASLH and SpaASLH/G46A/G109A structures, and SpaASLH/G109A (PDB: 6CWN, with the monosaccharide ligand removed) used as a search model for the SpaASLH/G109A structures. Following initial refinements, the 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAcOMe disaccharide and 4,6-Pyr-β-D-ManNAc-(1→4)-β-D-GlcNAc-(1→3)-4,6-Pyr-β-D-ManNAcOMe trisaccharide ligands were generated using the PRODRG2 Server and incorporated into their corresponding molecular models (39). All refinements and model building steps were performed using Coot (40) and Refmac5 from the CCP4 suite (34).
SpaSLH recognition of pyruvylated terminal SCWP units

Visualization and graphics

All figures depicting chemical structures or electron density were created using the UCSF Chimera Extensible Molecular Modeling System, developed by the Resource for Bio-computing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (41).

Data availability

The atomic coordinates and structure factors (7SV3, 7SV4, 7SV5, and 7SV6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

Supporting information—This article contains supporting information (27, 35).

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Abbreviations—The abbreviations used are: G1, groove 1 of SpaASLH; G2, groove 2 of SpaASLH; G3, groove 3 of SpaASLH; ITC, isothermal titration calorimetry; SapSLH, SLH domain trimer from the Bacillus anthracis S-layer protein SpaA.

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