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Distinct Affinity of Binding Sites for S-Layer Homologous Domains in *Clostridium thermocellum* and *Bacillus anthracis* Cell Envelopes

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Binding parameters were determined for the SLH (S-layer homologous) domains from the *Clostridium thermocellum* outer layer protein OlpB, from the *C. thermocellum* S-layer protein SlpA, and from the *Bacillus anthracis* S-layer proteins EA1 and Sap, using cell walls from *C. thermocellum* and *B. anthracis*. Each SLH domain bound to *C. thermocellum* and *B. anthracis* cell walls with a different K_D, ranging between 7.1 × 10^{-7} and 1.8 × 10^{-8} M. Cell wall binding sites for SLH domains displayed different binding specificities in *C. thermocellum* and *B. anthracis*. SLH-binding sites were not detected in cell walls of *Bacillus subtilis*. Cell walls of *C. thermocellum* lost their affinity for SLH domains after treatment with 48% hydrofluoric acid but not after treatment with formamide or dilute acid. A soluble component, extracted from *C. thermocellum* cells by sodium dodecyl sulfate treatment, bound the SLH domains from *C. thermocellum* but not those from *B. anthracis* proteins. A corresponding component was not found in *B. anthracis*.

The sequences of many bacterial cell surface proteins contain a conserved region termed the SLH (S-layer homologous) domain (7) which is composed of about 50- to 60-amino-acid segments usually reiterated threefold. SLH domains were shown to mediate binding of exocellular proteins to the cell surface in vivo and in vitro. In vivo, a mutant of *Thermus thermophilus* in which the SLH domain of the S-layer protein was deleted produced an S-layer which no longer bound to the cell surface (11). In vitro, the SLH domains of the *Clostridium thermocellum* outer layer protein OlpB and of the *C. thermocellum* S-layer protein SlpA bound to cell wall preparations (4, 5). The mode of attachment of SLH domains to the cell wall in *Bacillus stearothermophilus* PV72/p2 was proposed to be mediated by a secondary cell wall polysaccharide containing *N*-acetylglucosamine and *N*-acetylmannosamine in a molar ratio of 4:1 (12). In *C. thermocellum*, SLH domains were shown to bind not only to cell walls but also to a soluble cell envelope component which could be extracted by treating cells with sodium dodecyl sulfate (SDS) at 100°C (5). In this report, we compare the SLH-binding properties of cell envelope components from *Bacillus subtilis*, *C. thermocellum*, and *Bacillus anthracis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Wild-type *C. thermocellum* NCIB 10682 was grown anaerobically, at 60°C and without stirring, in complete CM3-3 medium (16) containing 5 g of cellobiose (Fluka AG) per liter. The *B. anthracis* plasmidless strain 9131, which is devoid of a capsule (1), and the *B. subtilis* wild-type strain JH642 (from J. A. Hoch) were grown aerobically at 37°C in LB medium (8). *Escherichia coli* TG1 (2), M15(pREP4) (18), and BL21(pREP4) (15) were used as cloning hosts and were grown at 37°C in LB medium. *Ticarcillin* (100 μg/ml) and kanamycin (25 μg/ml) were added, depending on the plasmids present in the host.

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Preparation of SLH polypeptides derived from *C. thermocellum* OlpB and SlpA and from *B. anthracis* EA1 and Sap. MalE-OlpB$_{1391-1664}$ (formerly termed MalE-ORF1p-C; (subscript numbers denote amino acids encompassed by the indicated protein) and SlpA$_{27-235}$ were purified from *E. coli* TG1(pCT1473) and *E. coli* M15(pREP4) harboring pCT1923, respectively, as described previously (4, 5). The expression plasmids pQE1473, pQEEA1, and pQESAP were obtained by subcloning appropriate restriction or PCR fragments into pQE30 or pQE31 (Qiaexpress kit; Qiagen). OlpB$_{1391-1664}$ and EA1$_{32-213}$ were purified from *E. coli* BL21(pREP4) harboring pQE1473 and pQEEA1, respectively, and Sap$_{32-211}$ was purified from *E. coli* M15(pREP4) harboring pQESAP. Each of the overproduced polypeptides was fused to six N-terminal His residues encoded by the vector, enabling purification by Ni^{2+} affinity chromatography (3) as described previously (4). For purification of EA1$_{32-213}$ and Sap$_{32-211}$ polypeptides, 40 mM imidazole was added to the wash buffer.

Cell wall preparation. Cell walls were isolated as described by Lemaire et al. (5). Briefly, whole cells were treated twice with boiling SDS and sonicated, and the pelleted insoluble material was treated once again with boiling SDS. The resulting cell wall preparation was washed five times with 20 mM sodium phosphate buffer (pH 7.5). The *meso*-diaminopimelate content, which is proportional to peptidoglycan content, was determined for each cell wall preparation by acid hydrolysis followed by amino acid analysis (5).

Affinity of SLH polypeptides for cell walls. Each SLH polypeptide was incubated at various concentrations (40 to 240 μg/ml) for 1 h at 37°C with shaking in 100 μl of 20 mM sodium phosphate buffer (pH 7.5) containing cell walls from *C. thermocellum* (containing 34.6 μM *meso*-diaminopimelate) or *B. anthracis* (containing 97.3 μM *meso*-diaminopimelate). For *B. subtilis*, cell walls (containing 251.5 μM *meso*-diaminopimelate) were incubated in the presence of 20 μg of each SLH polypeptide. Free SLH polypeptides were separated from bound SLH polypeptides by centrifugation twice at 40,000 × *g* for 20 min. The concentration of free SLH polypeptide was assayed in the pooled supernatants by using the micro bicinchoninic acid protein assay reagents (Pierce). The total amount of polypeptide present in the resuspended pellet fraction was assayed with the Coomassie blue reagent (Bio-Rad). The amount of bound SLH polypeptide was calculated by subtracting the amount of free SLH polypeptide remaining in the pellet suspension. Colorimetric determinations were converted to molar concentrations by reference to a standard consisting of a stock of the same polypeptide whose molarity was determined by UV absorption at 280 nm. Data points were fitted with one-site binding-type hyperbolas by nonlinear regression using the Prism program (GraphPad).

Effect of various extraction procedures on the SLH-binding ability of *C. thermocellum* cell walls. SDS-extracted cell walls were washed four times with distilled water and vacuum dried. Aliquots were then treated with formamide for 1 h at 100 or 150°C, with 25 mM glycine-HCl (pH 2.5) for 0.5 h at 100°C, with 0.1 M HCl for 0.5 h at 60°C, or with 48% hydrofluoric acid (HF) for 22 h at 4°C and finally washed with distilled water (12). Binding of MalE-OlpB$_{1391-1664}$ to native or treated cell walls was tested as described previously (5). After incubation in the presence of cell walls, bound and free polypeptides were separated by centrifugation. The supernatant constituted the soluble fraction. A wash fraction was obtained after washing cell walls with 50 mM Tris-HCl (pH 7.5). The

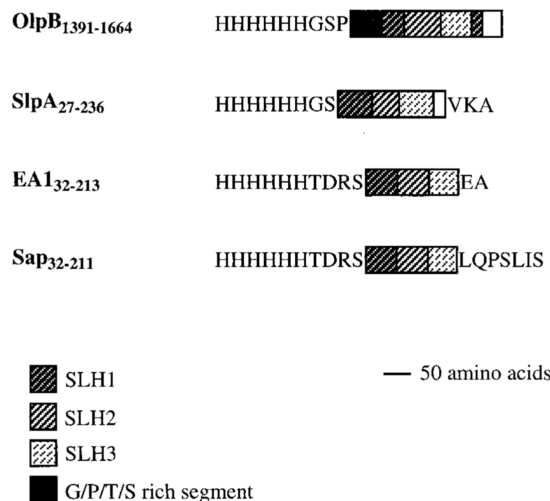


FIG. 1. Schematic representation of SLH polypeptides purified from *E. coli*. The triplicated segments of SLH domains extending between amino acids indicated by the numbers are shown by boxes. Amino acids encoded by the expression vector are indicated. The SLH domain of OlpB carries a circular permutation (6), resulting in the location of the N-terminal part of the first SLH segment at the C terminus of the third SLH segment.

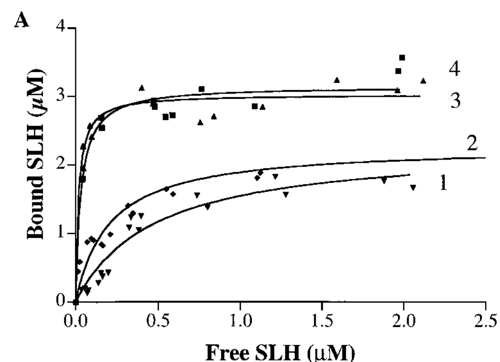
insoluble fraction consisted of material eluted from cell walls in the presence of hot SDS. Each fraction was analysed by SDS-polyacrylamide gel electrophoresis.

Binding of SLH polypeptides to the SDS-soluble component of *C. thermocellum* cell envelopes. *C. thermocellum* cells were treated for 15 min with SDS at 100°C, and the extracted material was subjected to SDS-polyacrylamide gel electrophoresis (5). Following transfer to nitrocellulose, blots were incubated with ^{125}I-labeled SLH polypeptides and autoradiographed (17).

RESULTS

Parameters of binding of different SLH domains to cell walls from *B. subtilis*, *C. thermocellum*, and *B. anthracis*. Four different SLH polypeptides were tested for binding to cell walls of *B. subtilis*, *C. thermocellum*, or *B. anthracis* (Fig. 1). $OlpB_{1391-1664}$ corresponds to the C-terminal region of the *C. thermocellum* outer layer protein OlpB (5). $SlpA_{27-235}$ contains the N-terminal SLH domain of the *C. thermocellum* S-layer protein SlpA (4). $EA1_{32-213}$ and Sap_{32-211} correspond to the N-terminal SLH domains of the two S-layer proteins EA1 and Sap of *B. anthracis* (1, 10). Binding was estimated from the amount of polypeptide cosedimenting with a known amount of cell walls, normalized for its content in *m*-diaminopimelate. The cell wall preparations consisted mostly of peptidoglycan and contained no significant amount of covalently associated protein.

The quantities of $OlpB_{1391-1664}$, $SlpA_{27-235}$, $EA1_{32-213}$, and Sap_{32-211} polypeptides cosedimenting with *B. subtilis* cell walls amounted to less than 2 mmol/mol of *meso*-diaminopimelate, indicating that *B. subtilis* cell walls do not have binding sites for *C. thermocellum* or *B. anthracis* SLH domains.



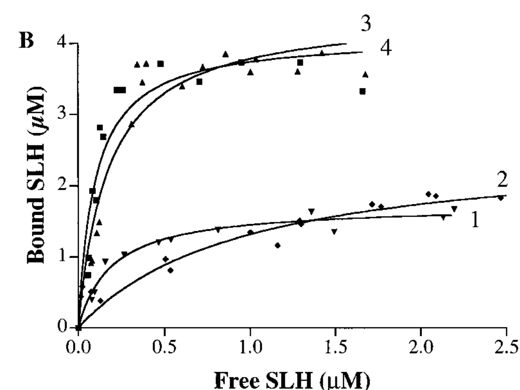


FIG. 2. Binding of SLH domains to cell walls of *C. thermocellum* containing 34.6 μM *meso*-diaminopimelate (A) and to cell walls of *B. anthracis* containing 97.3 μM *meso*-diaminopimelate (B). Curve 1, $OlpB_{1391-1664}$ (▼); curve 2, $SlpA_{27-235}$ (◆); curve 3, $EA1_{32-213}$ (▲); curve 4, Sap_{32-211} (■).

As shown in Fig. 2A and Table 1, *C. thermocellum* cell walls bound the *C. thermocellum* polypeptides $OlpB_{1391-1664}$ and $SlpA_{27-235}$ with the same capacity but with a 2.3-fold-higher affinity for $SlpA_{27-235}$ than for $OlpB_{1391-1664}$. *B. anthracis* polypeptides $EA1_{32-213}$ and Sap_{32-211} both were bound by *C. thermocellum* cell walls with a 1.4-fold-higher capacity and affinities that were an order of magnitude higher than observed for *C. thermocellum* polypeptides, the highest value being obtained for $EA1_{32-213}$ ($K_D = 1.8 \times 10^{-8}$ M).

B. anthracis cell walls displayed significant differences from

TABLE 1. Parameters of binding of $OlpB_{1391-1664}$, $SlpA_{27-235}$, $EA1_{32-213}$, and Sap_{32-211} polypeptides to *C. thermocellum* and *B. anthracis* cell walls

Protein	*C. thermocellum* cell walls		*B. anthracis* cell walls	
	K_D (M)	Capacity[a]	K_D (M)	Capacity
$OlpB_{1391-1664}$	4.8×10^{-7} ([2.7–6.9] $\times 10^{-7}$[b])	66 (55–78[b])	1.9×10^{-7} ([1.2–2.6] $\times 10^{-7}$)	18 (16–19)
$SlpA_{27-235}$	2.1×10^{-7} ([1.2–2.9] $\times 10^{-7}$)	66 (58–74)	7.1×10^{-7} ([3.7–10.4] $\times 10^{-7}$)	24 (20–27)
$EA1_{32-213}$	1.8×10^{-8} ([1.0–2.6] $\times 10^{-8}$)	88 (83–93)	1.8×10^{-7} ([0.9–2.6] $\times 10^{-7}$)	46 (40–51)
Sap_{32-211}	3.1×10^{-8} ([1.7–4.6] $\times 10^{-8}$)	91 (85–97)	1.0×10^{-7} ([0.5–1.5] $\times 10^{-7}$)	42 (36–48)

[a] Binding capacity of cell walls, expressed as millimoles of bound SLH domains per mole of *meso*-diaminopimelate (dap).
[b] 95% confidence interval.

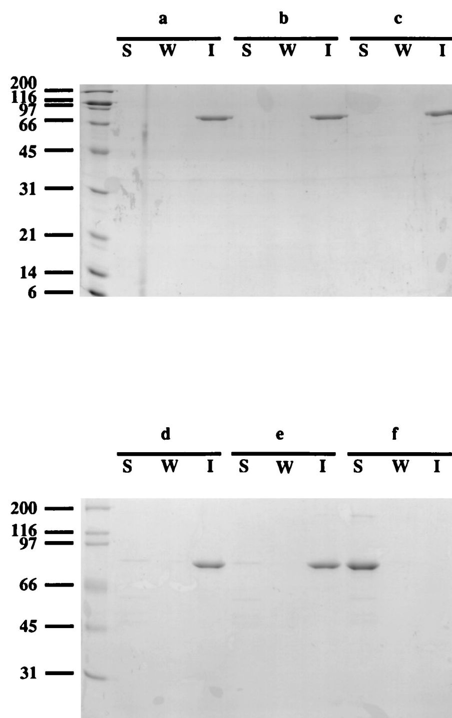


FIG. 3. Binding of MalE-OlpB$_{1391-1664}$ to *C. thermocellum* cell walls subjected to various extraction procedures. Native cell walls (a) were treated with formamide at 100°C (b) or 150°C (c), with 25 mM glycine-HCl (pH 2.5) (d), with 0.1 M HCl (e), or with 48% HF (f). Lanes: S, soluble fraction; W, wash fraction; I, insoluble fraction. The leftmost lane shows positions of molecular size markers (in kilodaltons).

C. thermocellum cell walls. The binding capacity was 1.8- and 2.4-fold (instead of 1.4-fold) greater for the *B. anthracis* than the *C. thermocellum* polypeptides (Fig. 2B and Table 1). The range of binding affinities was much narrower. In particular, K_D values for OlpB$_{1391-1664}$ and EA1$_{32-213}$ were similar, whereas they differed 27-fold in the case of binding to *C. thermocellum* cell walls. Furthermore, the binding affinity increased in the order OlpB $<$ SlpA $<<$ Sap $\leq$ EA1 in the case of *C. thermocellum* cell walls, whereas for *B. anthracis* cell walls the order was SlpA $<$ OlpB $\sim$ EA1 $\sim$ Sap.

Effects of different chemical treatments on the SLH-binding ability of *C. thermocellum* cell walls. It was proposed that in *B. stearothermophilus* PV72/p2, adhesion to the cell wall of the S-layer protein SbsB, which contains an SLH domain, was mediated by a secondary cell wall polysaccharide (12). This polysaccharide, containing *N*-acetylglucosamine and *N*-acetylmannosamine in a molar ratio of 4:1, could be extracted with dilute acid, formamide, or HF, leaving behind a peptidoglycan fraction which no longer bound SbsB. The same extraction procedures were applied to *C. thermocellum* cell walls, which were subsequently tested for the ability to bind MalE-OlpB$_{1391-1664}$, containing the SLH domain of OlpB grafted to MalE (5). As shown in Fig. 3, treatment with formamide (at 100 or 150°C) or with dilute acid (25 mM glycine-HCl [pH 2.5] or 0.1 M HCl) did not impair the ability of cell walls to bind MalE-OlpB$_{1391-1664}$. However, MalE-OlpB$_{1391-1664}$ no longer cosedimented with cell walls treated with 48% HF, showing that SLH binding was abolished. The HF-extracted material was analyzed for hexoses (14) after hydrolysis with 4 N trifluoroacetic acid for 4 h, yielding *N*-acetylglucosamine, glucose,

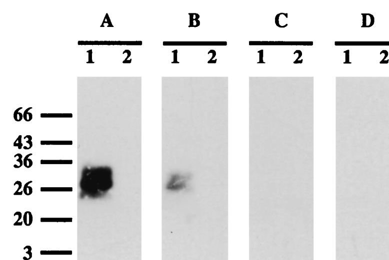


FIG. 4. Binding of OlpB$_{1391-1664}$ (A), SlpA$_{27-235}$ (B), EA1$_{32-213}$ (C), or Sap$_{32-211}$ (D) polypeptides labeled with ^{125}I to the SDS-soluble component extracted from *C. thermocellum* (lane 1) or *B. anthracis* (lane 2). The masses (in kilodaltons) and positions of migration of molecular size markers are indicated on the left.

galactose, and *N*-acetylmannosamine in a molar ratio of 1:6.4: 1.9:0.27.

Binding of SLH domains to components noncovalently linked to cell walls. As shown previously (5), a soluble component which binds the SLH domain of OlpB can be extracted from the surface of *C. thermocellum* by boiling cells in SDS. This component migrates in SDS-polyacrylamide gels like a 26/28-kDa polypeptide doublet. However, it is probably not a protein, since it is not extracted by phenol-chloroform and cannot be degraded with proteinase K or pronase (data not shown). Contrary to SLH-binding sites remaining on *C. thermocellum* cell walls treated with SDS, the 26/28-kDa component was sensitive to dilute acid (25 mM glycine-HCl buffer [pH 2.5]) (data not shown). Binding of the SLH domain of OlpB to the SDS-soluble component was demonstrated by transferring the component from SDS-polyacrylamide gels to nitrocellulose and by incubating the blots with ^{125}I-labeled MalE-OlpB$_{1391-1664}$ (5). The same experiment was performed with OlpB$_{1391-1664}$, SlpA$_{27-235}$, EA1$_{32-213}$, and Sap$_{32-211}$ polypeptides as ^{125}I-labeled probes. Figure 4 shows that the 26/28-kDa doublet bound OlpB$_{1391-1664}$ or SlpA$_{27-235}$ but neither EA1$_{32-213}$ nor Sap$_{32-211}$. To test whether a similar component could be detected in *B. anthracis*, the same experiment was repeated with SDS extracts of *B. anthracis* cells. No band was revealed with any of the ^{125}I-labeled polypeptides (Fig. 4).

DISCUSSION

Taken together, our results suggest that SLH-binding sites are different in different bacterial species and are not unique within the same species. Cell walls from *C. thermocellum* and *B. anthracis* can bind SLH domains from both bacteria, suggesting similar modes of interaction of SLH domains. However, comparison of binding parameters shows that SLH-binding sites are different in *C. thermocellum* and *B. anthracis*. None of the four SLH domains tested had the same affinity for cell walls from *C. thermocellum* and *B. anthracis*, and the binding specificities of the two cell wall preparations were different. Moreover, the different binding capacities of each cell wall preparation for different SLH polypeptides suggest that some SLH-binding sites are accessible to *B. anthracis* SLH domains but not to *C. thermocellum* SLH domains.

In addition to the SLH-binding sites of the SDS-insoluble cell walls, the envelope of *C. thermocellum* contains an SDS-soluble component that binds SLH domains (5). In contrast to *C. thermocellum* cell walls, it is not able to bind the SLH domains from the *B. anthracis* S-layer proteins EA1 and Sap. No similar component could be extracted from *B. anthracis* cells, suggesting that it is specific for *C. thermocellum*.

The SLH-binding site which is soluble in SDS is nonco-

valently linked to the cell wall. This finding is compatible with the hypothesis that this component may be located within the outer layer surrounding the S-layer of *C. thermocellum*. Indeed, OlpB, which was shown to be located in the outer layer (5), interacted more strongly with the SDS-soluble component but more weakly with cell walls than SlpA which was localized in the cell wall-associated S-layer (4). Thus, the presence of SLH-binding components with different specificities may afford the possibility for the bacterium to target exocellular proteins to different locations on the bacterial cell surface.

Cell walls of *B. subtilis* did not contain detectable binding sites for SLH domains. This is not unexpected, since sequence analysis of the *B. subtilis* genome did not reveal any gene putatively encoding a cell surface polypeptide containing an SLH domain. Therefore, SLH-binding components are probably restricted to the cell surface of bacteria secreting cell-associated proteins which carried SLH domains.

Our results strongly suggest that a secondary cell wall polymer is responsible for binding SLH domains to *C. thermocellum* cell walls. Treatment of *C. thermocellum* cell walls with 48% HF abolished binding of the SLH domain of OlpB, suggesting that a secondary polysaccharide may be involved. Indeed, material containing *N*-acetylglucosamine, glucose, galactose, and *N*-acetylmannosamine was found in the HF extract which may correspond, in part, to the binding target of SLH domains. A similar effect of HF on the SLH-binding capacity of *B. anthracis* cell walls has been reported (9). Conflicting data were reported for the binding of the S-layer protein SbsB to cell walls of *B. stearothermophilus* PV72/p2. Treatment of *B. stearothermophilus* cell walls with HF was reported to abolish binding of SbsB and to extract a polysaccharide composed of *N*-acetylglucosamine and *N*-acetylmannosamine in a 4:1 molar ratio. The latter component was shown to bind to SbsB (12). More recently, it was reported that SbsB binds to HF-extracted *B. stearothermophilus* cell walls, provided that its SLH domain is intact (13). If confirmed, this finding suggests that SLH domains may have yet a different binding target (i.e., pure peptidoglycan) in *B. stearothermophilus*. Further studies with purified SLH-binding components will be required to understand the molecular basis for the affinity and specificity of interaction between SLH domains and their binding targets.

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