Identification of a region responsible for binding to the cell wall within the S-layer protein of *Clostridium thermocellum*

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The protomer forming the S-layer of *Clostridium thermocellum* was identified as a 140 kDa protein which was non-covalently bound to the cell wall. Cloning and sequencing of the corresponding gene revealed an open reading frame of 3108 nucleotides encoding a polypeptide of 1036 amino acids, termed SlpA. The amino acid composition of SlpA matches the composition of a previously described exocellular glycoprotein. SlpA shared extensive similarity with the S-layer protein of *Bacillus sphaericus* and with the outer wall protein of *Bacillus brevis*. In addition, the amino-terminal region of SlpA contained a segment presenting similarities with segments termed SLH (S-layer homologous), which are found in several bacterial exoproteins. A polypeptide of 209 residues comprising this segment was shown to bind to cell walls extracted from *C. thermocellum* cells.

Keywords: S-layer protein, *Clostridium thermocellum*, S-layer homologous (SLH) domain

INTRODUCTION

Clostridium thermocellum, a thermophilic, anaerobic bacterium, is best known for producing highly active multienzyme cellulase complexes termed cellulosomes (Johnson *et al.*, 1982; Lamed *et al.*, 1983). Electron micrographs show that the cytoplasmic membrane of *C. thermocellum* is surrounded by three layers: a relatively thin peptidoglycan layer, a regularly ordered S-layer, and an amorphous outer layer (Lamed & Bayer, 1988; Nolte, 1992). Cellulosomes have been located in protuberances formed by the outer layer (Bayer & Lamed, 1986; Bayer *et al.*, 1985; Nolte & Mayer, 1989). They are associated with a set of polypeptides thought to be responsible for the attachment of cellulosomes and individual cellulases to the surface of the cells (Fujino *et al.*, 1993; Leibovitz & Béguin, 1996; Salameitou *et al.*, 1994a, b). These polypeptides contain a threefold-reiterated segment, termed the S-layer homologous (SLH) domain, which has been found in a number of S-layer proteins and in cell-bound exoenzymes degrading

polysaccharides (Lupas *et al.*, 1994; Matuschek *et al.*, 1994). SLH domains appear to mediate attachment of proteins to the cell wall (Lemaire *et al.*, 1995; Olabarria *et al.*, 1996) as well as interactions with other SLH domains (Lemaire *et al.*, 1995).

This prompted us to investigate whether SLH domains may also be involved in the interaction of the S-layer with the cell wall of *C. thermocellum*. This paper reports the identification of the protomer forming the S-layer of *C. thermocellum*, the demonstration that it is non-covalently bound to the cell wall, and the cloning and sequencing of the corresponding gene. A polypeptide derived from the amino-terminal region of the protein and harbouring a segment distantly related to SLH domains was found to bind to the *C. thermocellum* cell wall.

METHODS

Bacterial strains and culture conditions. *C. thermocellum* NCIMB 10682 was grown anaerobically with gentle stirring at 60 °C in complete CM3-3 medium (Tailliez *et al.*, 1989) containing 5 g cellobiose l⁻¹ (Fluka AG). *Escherichia coli* strains TG1 (Gibson, 1984) and M15(pREP4) were used as cloning hosts and were grown at 37 °C in LB medium (Maniatis *et al.*, 1982). Ticarcillin (100 µg ml⁻¹) and kanamycin (25 µg ml⁻¹) were added, depending on the plasmids present in the host.

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Abbreviation: SLH, S-layer homologous.

The GenBank accession number for the sequence reported in this paper is U79117.

Preparation of Triton-X-100-treated cell envelopes of *C. thermocellum*. *C. thermocellum* cells from 50 ml mid-exponential-phase cultures (OD_{600} 1–2; Beckman model 34 spectrophotometer) were harvested by centrifugation, washed with 0.15 M NaCl, and resuspended in 5 ml 50 mM Tris/HCl (pH 7.5) containing 150 mM NaCl (buffer A). Four millilitres of the suspension was disrupted by sonication, intact cells were removed by centrifuging twice for 5 min at 1900 g_{max} and envelopes were pelleted by centrifuging for 20 min at 39 000 g_{max} . Triton-X-100-treated envelopes were prepared at room temperature by resuspending and centrifuging the pellet (20 min at 39 000 g_{max}) successively in 1 ml 1 M NaCl, 1 ml buffer A and 1 ml buffer A containing 1% (v/v) Triton X-100. The final pellet was resuspended in 1 ml buffer A.

Treatment of cell envelopes with lysozyme. Triton-X-100-treated cell envelopes corresponding to 2 ml original culture were incubated for 20 min at 37 °C in 100 μ l 50 mM Tris/HCl (pH 7.5) containing or not containing 10 μ g lysozyme and centrifuged for 30 min at 20 000 g . The insoluble fraction was extracted by heating at 100 °C with 100 μ l sample buffer (Laemmli, 1970). Ten microlitres of the supernatant and of the insoluble fraction extracted by sample buffer were analysed by SDS-PAGE (Laemmli, 1970).

Electron microscopy of cell envelopes. Triton-X-100-treated cell envelopes were diluted 1:100 (v/v) in 0.1 M ammonium acetate, 0.05 M ammonium bicarbonate, 0.1 mM EDTA (pH 7.2) containing 6% glycerol. The envelopes were mixed thoroughly and spread over freshly cleaved mica. The samples were rotary-shadowed with platinum as previously described by Bacallà *et al.* (1991). Replicas were observed with a Philips CM12 electron microscope operating at 60 kV.

Sequencing of the amino-terminus and of internal peptides of the *C. thermocellum* S-layer protein. For amino-terminal sequencing, cell envelopes containing approximately 5–10 μ g S-layer protein were subjected to SDS-PAGE and transferred onto a hydrophobic PVDF membrane (Problott; Applied Biosystems) using the conditions recommended by the manufacturer. The 140 kDa band was stained with 0.003% amido black (Aldrich) and cut out, and the amino-terminal sequence of the polypeptide was determined by the Edman method, using a model 473 A sequencer (Applied Biosystems). For sequencing internal peptides, 200 pmol of the protein was loaded on an SDS-PAGE gel. The 140 kDa band was cut out after staining with amido black and digested *in situ* with pig trypsin. Peptides were separated by C_{18} reverse-phase chromatography using a water/acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. Two peptides having a high absorbance at 280 nm were sequenced as described above.

DNA manipulations. *C. thermocellum* genomic DNA was purified by the method of Quiviger *et al.* (1982). Other DNA manipulations were performed as described by Ausubel *et al.* (1990). Restriction enzymes were used as recommended by the suppliers.

Cloning and sequencing of the gene encoding the S-layer protein of *C. thermocellum*. The degenerate oligonucleotide GAY GAT GAT GCI GTI GTI GAY GCI TGG GG, matching the internal peptide sequence DDDAVVDAWG, was labelled with [γ - 32 P]ATP and T4 polynucleotide kinase and used as a hybridization probe. Southern blots were performed on *C. thermocellum* DNA cut with various restriction enzymes, using increasingly stringent washing conditions. Upon washing blots twice for 30 min at 45 °C with $1 \times$ SSC (0.15 M NaCl; 0.015 M sodium citrate), a major and a minor band were present in all digests. The 1.33 kb region of a *Hind*III digest of *C. thermocellum* genomic DNA corresponding to the

stronger hybridizing *Hind*III fragment was extracted from an agarose gel and cloned into *Hind*III- and phosphatase-treated pTZ19R. One hundred and twenty cultures were started from independent recombinant colonies, and minipreparations of plasmid DNA were made from pools of three cultures each. Plasmid DNA was subjected to dot-blot hybridization, yielding one positive pool, from which one clone was isolated which contained a 1.33 kb DNA fragment encoding the previously sequenced oligopeptide. The remaining part of the sequence was obtained after cloning a 2.9 kb *Pst*I fragment bracketing the *Hind*III fragment, and a 0.85 kb *Hind*III fragment overlapping the latter (Fig. 4). DNA sequencing was performed on double-stranded templates using the Thermo-sequenase kit (Amersham) and synthetic oligonucleotides provided by Eurogentec or Genset.

Synthesis and purification of the SlpA_{27–235} polypeptide comprising the amino-terminal region of SlpA. The DNA segment encoding the region extending from codon 27 (beginning of the mature protein) to codon 235 of the coding sequence was amplified by PCR. Primers were TTT GCT GGA TCC GAT TCA TTC AGC TAT GAA AAA G and CAT CCA AAG CTT TTA CTT CCT TGA TGG CTT T, which provided *Bam*HI and *Hind*III restriction sites flanking the 5' and 3' ends, respectively, of the fragment (Fig. 4). The amplified fragment was cloned into *Bam*HI- and *Hind*III-digested pQE-30 yielding pCT1923. In this construct, the coding sequence of the *slpA* fragment is preceded by six His codons provided by the vector, enabling purification of the fusion polypeptide by Ni²⁺ affinity chromatography (Janknecht *et al.*, 1991). The sequence of the insert was verified, and the plasmid was transferred to *E. coli* M15(pREP4) (Farabaugh, 1978; Villarejo & Zabin, 1974) (Qiaexpress kit; Qiagen). A 1 l culture of the resulting strain was grown to OD_{600} 0.5 and induced overnight in the presence of 0.5 mM IPTG. Cells were centrifuged and resuspended in 25 ml 50 mM Tris/HCl (pH 7.5) and disrupted in an Aminco French pressure cell at 100 MPa. The extract was centrifuged for 20 min at 25 000 g , and the cleared supernatant was loaded on an 8 ml Ni²⁺-nitrilotriacetic column (Qiagen) equilibrated with 50 mM Tris/HCl (pH 7.5). The column was washed with the same buffer and eluted with the same buffer containing 250 mM imidazole. The eluted fractions were pooled and dialysed against 50 mM Tris/HCl (pH 7.5). The final yield of purified polypeptide was 48 mg (1 culture)^{–1}.

Interaction of the SlpA_{27–235} polypeptide with the cell wall of *C. thermocellum*. *C. thermocellum* cell walls were obtained after washing cell envelopes in the presence of boiling SDS, and binding experiments were performed by incubating and co-sedimenting the SlpA_{27–235} polypeptide with the cell wall (Lemaire *et al.*, 1995). Amino acid and glucosamine analysis of the cell wall fraction, with and without adsorbed SlpA_{27–235}, was performed as described previously (Lemaire *et al.*, 1995).

RESULTS

Identification of the S-layer protein of *C. thermocellum*

Two major polypeptides with molecular masses of 50 and 140 kDa co-sediment with cell envelopes of *C. thermocellum*. The 50 kDa polypeptide was partially solubilized in the presence of 1 M NaCl, and totally solubilized in the presence of 1% Triton X-100, leaving the 140 kDa polypeptide, which was insoluble in the presence of Triton X-100. Both proteins were extracted

Fig. 1. SDS-PAGE analysis of *C. thermocellum* cell envelopes. Cell envelopes were prepared as described in Methods and washed successively with 1 M NaCl (a), 50 mM Tris/HCl (pH 7.5) containing 150 mM NaCl (b) and 50 mM Tris/HCl (pH 7.5) containing 150 mM NaCl and 1% Triton X-100 (c). Aliquots equivalent to 100 μ l original culture from the soluble (S) and insoluble (I) fraction obtained after each washing step were analysed by SDS-PAGE (Laemmli, 1970). The size in kDa of molecular mass markers (M) is indicated on the left.

by hot SDS-PAGE sample buffer (Fig. 1). Electron microscopic examination of the preparation before (data not shown) and after (Fig. 2) Triton X-100 extraction showed the presence of cell envelope fragments displaying a regular, oblique arrangement of subunits, typical of S-layer lattices. The 140 kDa polypeptide was by far the most abundant protein which could be solubilized by hot SDS from the Triton-X-100-extracted preparation. Since it is known from amino acid analysis that the insoluble cell wall fraction obtained after washing envelopes with hot SDS is devoid of associated protein (Lemaire *et al.*, 1995), this suggests that the 140 kDa polypeptide corresponds to the S-layer protomer.

Association of the S-layer protein with the cell wall

Fig. 3 shows that the 140 kDa S-layer protein can be solubilized by incubating Triton-X-100-treated cell envelopes in the presence of lysozyme, indicating that it is probably associated, directly or indirectly, with the peptidoglycan layer. Little solubilization occurred upon incubating the preparation at 37 °C for the same period of time in the absence of lysozyme, although upon longer incubation (several hours), the S-layer protein was ultimately released spontaneously in the supernatant (data not shown).

Cloning and sequencing of the *slpA* gene encoding the S-layer protein

A 1.33 kb *Hind*III fragment of the *slpA* gene encoding the S-layer protein was cloned by hybridization with a degenerate oligonucleotide probe derived from the

Fig. 2. Electron micrograph of a cell envelope fragment treated with Triton X-100 and stained by rotary shadowing. The region shown in brackets in the top part of the figure is shown enlarged in the bottom part. Bar, 100 nm.

sequence of an internal peptide of the protein. Fragments covering the rest of the gene were cloned by 'chromosome walking' on either side of the cloned fragment. The nucleotide sequence shows the presence of an open reading frame encoding a polypeptide of 1036 residues, with a molecular mass of 113260 Da. The predicted amino acid sequence matches the sequence of the peptide used to design the oligonucleotide probe, as well as that of another internal peptide. The sequence encoding the amino-terminus of the protein isolated from *C. thermocellum* starts at the 27th codon of the coding sequence. The composition of the mature protein is similar to the composition reported by Lamed & Bayer (1988) for a 130 kDa glycoprotein present in the culture supernatant and on the cell surface of *C. thermocellum* (Table 1).

Motifs identified within the sequence of the SlpA polypeptide

A summary of the features identified within the sequence of SlpA is presented in Fig. 4. SlpA shares a conserved core of about 450 residues with two other S-layer proteins, the outer wall protein of *Bacillus brevis* (GenBank accession no. P09333) (Tsuboi *et al.*, 1986), and the S-layer protein of *Bacillus sphaericus* (GenBank accession no. P38537) (Bowditch *et al.*, 1989). The three

Fig. 3. Solubilization of the S-layer protein upon lysozyme treatment of cell envelopes. Triton-X-100-treated cell envelopes were incubated in the presence or absence of lysozyme as described in Methods. The solubilized (S) and insoluble (I) fractions were analysed by SDS-PAGE after extraction of the latter with SDS-containing sample buffer. (a) Envelopes incubated without lysozyme; (b) envelopes incubated in the presence of lysozyme. The migration of molecular size markers (in kDa) is indicated on the right.

Table 1. Amino acid composition of SlpA and of the DE-130 glycoconjugate analysed by Lamed & Bayer (1988)

The amino acid composition is expressed as residues/100 residues. ND, Not determined.

Amino acid	SlpA*	DE-130
Asx	13.37	13.6
Thr	8.22	7.7
Ser	5.84	5.8
Glx	11.98	13.6
Pro	2.57	2.7
Gly	6.44	6.6
Ala	8.42	8.5
Cys	0.10	0.0
Val	10.59	7.8
Met	0.79	1.0
Ile	5.94	4.3
Leu	6.93	6.7
Tyr	4.36	4.8
Phe	2.97	3.2
His	0.00	0.3
Lys	9.41	8.6
Arg	1.68	1.7
Trp	0.40	ND

* As predicted from the sequence of the mature protein.

sequences share a reiterated motif which is repeated five times in each polypeptide. The first four reiterated motifs occur at intervals of about 100 residues, while the fifth one is located some 220 residues downstream from the fourth one. The amino-terminal region contains three segments resembling SLH domains. However, the similarity is not very strong. In particular, most other SLH segments contain the signature (I,L,V,M,A)(T,S)R (A,S,Q)(E,D,Q) (most frequently found residues are in bold) (Fig. 5 and Lupas *et al.*, 1994), but this motif is absent from the second repeat of SlpA, and barely recognizable in the first and third repeats.

Binding to the *C. thermocellum* cell wall of a 209-residue polypeptide comprising the SLH domain of SlpA

By analogy with other SLH domains, it was surmised that the SLH-like segment of SlpA may mediate attachment of the S-layer to the cell wall. The SlpA₂₇₋₂₃₅ polypeptide, containing the SLH-like region, was constructed by fusing a 209-residue segment extending downstream from the amino-terminus of mature SlpA to the His₆ tag encoded by the pQE-30 expression vector (Fig. 4). After purification by Ni²⁺ affinity chromatography, SlpA₂₇₋₂₃₅ was tested for its capacity to bind to the SDS-extracted cell wall fraction from *C. thermocellum*. As shown in Fig. 6, SlpA₂₇₋₂₃₅ was able to bind to the cell wall fraction. Little if any SlpA₂₇₋₂₃₅ was released upon washing the cell wall pellet. No SlpA₂₇₋₂₃₅ was pelleted in the absence of the cell wall fraction (data not shown). To show that the interaction between SlpA₂₇₋₂₃₅ and the cell wall fraction was specific, the experiment was repeated with a mixture containing a fourfold excess of a crude cytoplasmic extract of *E. coli* proteins added to SlpA₂₇₋₂₃₅. Although some *E. coli* polypeptides marginally adsorbed to the cell wall fraction, SlpA₂₇₋₂₃₅ was by far the major polypeptide co-sedimenting with the cell wall pellet (Fig. 6). To estimate the amount of bound SlpA₂₇₋₂₃₅, amino acid analysis was performed on the purified polypeptide and on the cell wall fraction containing or not containing the co-adsorbed polypeptide. Based on the glucosamine and phenylalanine content of the samples, 0.066 mol SlpA₂₇₋₂₃₅ was bound per mol glucosamine plus muramic acid present in the pelleted fraction (muramic acid is converted into glucosamine during the acid hydrolysis performed prior to amino acid analysis).

DISCUSSION

The *C. thermocellum* cell envelope contains two major components, with molecular masses of about 50 and 140 kDa. The 50 kDa component can be solubilized in the presence of Triton X-100, suggesting that it may be included within the cytoplasmic membrane. The 140 kDa protein, now termed SlpA, is the protomer forming the S-layer surrounding the cell. SlpA is released in large quantities into the medium of cultures grown to stationary phase (unpublished observations) and appears to be identical to DE-130, the major non-

Fig. 4. Map of the *slpA* region of *C. thermocellum* and construction of pCT1923 encoding residues 27–235 of SlpA fused to six His residues. The positions of the segments encoding the various regions identified within SlpA are indicated by boxes of different patterns, together with similar regions found in *B. brevis* outer wall protein (OWP) and *B. sphaericus* S-layer protein (Slp). Residues of *slpA* that have been modified to introduce a *Bam*HI site are shown in bold type. Bg, *Bgl*II; H, *Hind*III; E, *Eco*RI; P, *Pst*I; Xh, *Xho*I.

SlpA SLH1	27	-----DSFSYKEEA-EILYRIGLYKG-----TSETYEYVENLEGKLDRTQGVVMLRLFGQEDDALEIPMDEAAQTLAA	93
SlpA SLH2	94	KFKDAADIADNAQRQVAYAVERGIVK-----YPDGTLEENAD--LNLGAFCSLLIQQL-----	145
SlpA SLH3	146	GYDGD-----FVFDEAAAYKIQEFGGLTA-----EQAEAFNNKNG--INRDSMVGIAFSATQAVYKATGKTVIEVLVENG	213
B. sph. SLH1	32	QLNDFNKISGYAKRAVQSLVDAGVIGQ-----DANGNFNPLKT--ISRAEAATIFTNAIELEAEGDV-----	91
B. sph. SLH2	92	NEKLVKA-DAWYYDATAATVENGIFEG-----VSATEFAFNKQ--LTRSEAAKILVDAFELEGEGLDLS-----	151
B. sph. SLH3	152	EFADASTVKPWAKSYLEIAVANGVIRGSEANGKTNLPNAP--ITRQDFAVVFSRTIENVDATPKVDKIEEVVDAKT	225
SdbA SLH1	453	TEALIGS-YAWAKKQIEVLASKGVING-----TSDTTFTPQAD--ITRADFMILLVKAIGLTAEVTS-----	511
SdbA SLH2	512	NEDIVSE-KDYIYFYVGIAGEIGITTC-----VGNKNFNEKAK--ITRQDMMLVLTNARIAGKISSTGTRADVE---	578
SdbA SLH3	579	RFSEKDKIASYAVEGVATLVKEGLVVC-----SGDIINERGN--ASRAELAAIYKIYYK	631

Fig. 5. Alignment of the SLH segments of *C. thermocellum* SlpA (SlpA SLH1, SLH2 and SLH3) with the three SLH segments of the *B. sphaericus* S-layer protein (B. sph. SLH1, SLH2 and SLH3) and with the three SLH segments of *C. thermocellum* SdbA (SdbA SLH1, SLH2 and SLH3) (Leibovitz & Béguin, 1996) (GenBank accession no. U49980). Boxes and shading indicate residues that are identical or similar in the majority of the segments, respectively.

cellulosomal protein identified in culture supernatants of *C. thermocellum* YS (Lamed & Bayer, 1988). Although the DE-130 component was not identified as an S-layer protein, its amino acid composition closely matched that predicted for SlpA, and antibody staining showed that it was indeed localized on the surface of *C. thermocellum* (Lamed & Bayer, 1988). DE-130 contains about 10% O-linked carbohydrate. However, the carbohydrate chains differ from those present in the cellosome-integrating protein CipA (formerly S_L or S1), since, unlike CipA, DE-130 does not bind to *Griffonia simplicifolia* lectin (Lamed & Bayer, 1988). The presence of carbohydrate may explain why SlpA/DE-130 migrates slower in SDS-PAGE than expected from the molecular mass of 110513 Da predicted from the sequence of the *slpA* gene. In addition to contributing to the mass of glycoproteins, glycosylation is known to

result in anomalous retardation in SDS-PAGE, due to reduced binding of SDS.

C. thermocellum SlpA, the outer wall protein of *B. brevis* and the S-layer protein of *B. sphaericus* form a family of S-layer proteins with a related core. The core sequence is punctuated by reiterated motifs. Speculations about the role of these motifs are premature, but it is tempting to correlate their presence at regular intervals with the structural constraints deriving from the formation of a regular lattice. The spacing between the fourth and the fifth repeat is nearly twice that observed between the other repeats, as if one repeat had been lost or altered beyond recognition in the course of evolution.

Maintenance of the S-layer of *C. thermocellum* depends on the integrity of the underlying peptidoglycan. This

Fig. 6. Binding of the SlpA₂₇₋₂₃₅ polypeptide to the cell wall fraction of *C. thermocellum*. (a) SDS-extracted cell wall fraction C4 (0.3 ml) (Lemaire *et al.*, 1995), corresponding to 15 ml original mid-exponential-phase culture of *C. thermocellum*, and 320 µg SlpA₂₇₋₂₃₅ in a total volume of 0.5 ml 50 mM Tris/HCl (pH 7.5). (b) As (a), plus 1.2 mg protein from a crude cytoplasmic extract of *E. coli* TG1. (c) Cell wall fraction plus cytoplasmic extract alone. All mixtures in (a–c) were incubated for 4 h at 37 °C and centrifuged at 40 000 *g* for 20 min. The supernatant yielded fraction S (soluble). Fraction W (wash) was obtained after washing the pellet in 0.5 ml of the same buffer. The washed pellet was extracted with 50 µl sample buffer, yielding fraction I (insoluble); 5 µl of each sample was analysed by SDS-PAGE.

indicates that SlpA must interact non-covalently with the cell wall. The amino-terminal region of SlpA was able to bind independently to hot-SDS-extracted cell envelopes of *C. thermocellum*. Thus, we suggest that this region participates in anchoring the S-layer to the cell wall. Sequence comparison shows that it is distantly related to previously identified SLH domains. These domains, which are present in numerous bacterial exoproteins, have been shown to bind *in vitro* (Lemaire *et al.*, 1995) and *in vivo* (Olabarriá *et al.*, 1996) to bacterial cell walls. By cell wall, we mean peptidoglycan and non-protein polymers that may be associated with it (Doyle, 1992), since experiments reported in this paper or in the above references do not discriminate between the two. Indeed, it is debated whether cell-wall-associated S-layer proteins attach to peptidoglycan, as proposed by Lupas *et al.* (1994), or to a peptidoglycan-associated polysaccharide, possibly containing glucosamine or *N*-acetylglucosamine (Hastie & Brinton, 1979; Sára *et al.*, 1996).

In spite of similarities, the sequence and the properties of the amino-terminal region of SlpA differ significantly from those of the more canonical SLH domains, such as those found in several proteins of *C. thermocellum*, e.g. OlpB, which are involved in attachment of cellulosomes or individual cellulases to the cell surface (Fujino *et al.*, 1993; Leibovitz & Béguin, 1996; Salamitou *et al.*, 1994a, b). Sequence differences are reflected in differences in immunological and biochemical properties between OlpB and SlpA. Antibodies directed against the

SLH segments present at the carboxy-terminus of OlpB cross-react with SLH segments present in three other polypeptides, but not with SlpA (Lemaire *et al.*, 1995). In addition, the SLH segments of OlpB not only bind to the cell surface of *C. thermocellum*, but also interact *in vitro* with SLH domains borne by other proteins (Lemaire *et al.*, 1995). In contrast, the SLH repeats of OlpB do not bind to SlpA (Lemaire *et al.*, 1995). Once structural data become available, it will be interesting to see whether binding of SLH domains to the cell wall involves residues that are conserved in SlpA and, conversely, whether homologous SLH–SLH interaction depends on residues that are absent from SlpA.

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REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1990). *Current Protocols in Molecular Biology*. New York: Greene Publishing and Wiley Interscience.
- Baccalà, R., Guilbert, B., Labrousse, H., Avrameas, S. & Gounon, P. (1991). Visualization of natural autoantibody polyreactivity by rotary metal-shadowing electron microscopy. *Res Immunol* 142, 299–312.
- Bayer, E. A. & Lamed, R. (1986). Ultrastructure of the cell surface cellulosome of *Clostridium thermocellum* and its interaction with cellulose. *J Bacteriol* 167, 828–836.
- Bayer, E. A., Setter, E. & Lamed, R. (1985). Organization and distribution of the cellulosome in *Clostridium thermocellum*. *J Bacteriol* 163, 552–559.
- Bowditch, R. D., Baumann, P. & Yousten, A. A. (1989). Cloning and sequencing of the gene encoding a 125-kilodalton surface-layer protein from *Bacillus sphaericus* 2362 and a related cryptic gene. *J Bacteriol* 171, 4178–4188.
- Doyle, R. J. (1992). Cell walls of bacteria. In *Encyclopedia of Microbiology*, pp. 479–493. Edited by J. Lederberg. New York: Academic Press.
- Farabaugh, P. J. (1978). Sequence of the *lacI* gene. *Nature* 274, 765–769.
- Fujino, T., Béguin, P. & Aubert, J.-P. (1993). Organization of a *Clostridium thermocellum* gene cluster encoding the cellulosomal scaffolding protein CipA and a protein possibly involved in the attachment of the cellulosome to the cell surface. *J Bacteriol* 175, 1891–1899.
- Gibson, T. J. (1984). *Studies on the Epstein–Barr virus genome*. PhD thesis, University of Cambridge.
- Hastie, A. T. & Brinton, C. C., Jr (1979). Specific interaction of the tetragonally arrayed protein layer of *Bacillus sphaericus* with its peptidoglycan sacculus. *J Bacteriol* 138, 1010–1021.

- Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R. A., Nordheim, A. & Stunnenberg, H. G. (1991). Rapid and efficient purification of native histidine-tagged protein expressed by recombinant vaccinia virus. *Proc Natl Acad Sci USA* **88**, 8972–8976.
- Johnson, E. A., Sakajoh, M., Halliwell, G., Madia, A. & Demain, A. L. (1982). Saccharification of complex cellulosic substrates by the cellulase system from *Clostridium thermocellum*. *Appl Environ Microbiol* **43**, 1125–1132.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lamed, R. & Bayer, E. A. (1988). The cellulosome concept: exocellular/extracellular enzyme reactor centers for efficient binding and cellulolysis. In *Biochemistry and Genetics of Cellulose Degradation* (FEMS Symposium no. 43), pp. 101–116. Edited by J.-P. Aubert, P. Béguin & J. Millet. London & New York: Academic Press.
- Lamed, R., Setter, E., Kenig, R. & Bayer, E. A. (1983). The cellulosome: a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. *Biotechnol Bioeng Symp* **13**, 163–181.
- Leibovitz, E. & Béguin, P. (1996). A new type of cohesin domain that specifically binds the dockerin domain of the *Clostridium thermocellum* cellulosome-integrating protein CipA. *J Bacteriol* **178**, 3077–3084.
- Lemaire, M., Ohayon, H., Gounon, P., Fujino, T. & Béguin, P. (1995). OlpB, a new outer layer protein of *Clostridium thermocellum*, and binding of its S-layer-like domain to components of the cell envelope. *J Bacteriol* **177**, 2451–2459.
- Lupas, A., Engelhardt, H., Peters, J., Santarius, U., Volker, S. & Baumeister, W. (1994). Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. *J Bacteriol* **176**, 1224–1233.
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Matuschek, M., Burchhardt, G., Sahm, K. & Bahl, H. (1994). Pullulanase of *Thermoanaerobacter thermosulfurigenes* EM1 (*Clostridium thermosulfurogenes*): molecular analysis of the gene, composite structure of the enzyme, and a common model for its attachment to the cell surface. *J Bacteriol* **176**, 3295–3302.
- Nolte, A. (1992). *Ultrastrukturelle, enzymkinetische und immuno- cytochemische Untersuchungen der extrazellulären Cellulase von Clostridium thermocellum JW20*. PhD thesis, Georg-August-Universität Göttingen.
- Nolte, A. & Mayer, F. (1989). Localization and immunological characterization of the cellulolytic enzyme system in *Clostridium thermocellum*. *FEMS Microbiol Lett* **61**, 65–72.
- Olabbarrá, G., Carrascosa, J. L., de Pedro, M. A. & Berenguer, J. (1996). A conserved motif in S-layer proteins is involved in peptidoglycan binding in *Thermus thermophilus*. *J Bacteriol* **178**, 4765–4772.
- Quiviger, B., Franche, C., Lutfalla, G. D. R., Haselkorn, R. & Elmerich, C. (1982). Cloning of a nitrogen fixation (*nif*) gene cluster of *Azospirillum brasilense*. *Biochimie* **64**, 495–502.
- Salamitou, S., Lemaire, M., Fujino, T., Ohayon, H., Gounon, P., Béguin, P. & Aubert, J.-P. (1994a). Subcellular localization of *Clostridium thermocellum* ORF3p, a protein carrying a receptor for the docking sequence borne by the catalytic components of the cellulosome. *J Bacteriol* **176**, 2828–2834.
- Salamitou, S., Raynaud, O., Lemaire, M., Coughlan, M., Béguin, P. & Aubert, J.-P. (1994b). Recognition specificity of the duplicated segments present in *Clostridium thermocellum* endoglucanase CelD and in the cellulosome-integrating protein CipA. *J Bacteriol* **176**, 2822–2827.
- Sára, M., Kuen, B., Mayer, H. F., Mandl, F., Schuster, K. C. & Sleytr, U. B. (1996). Dynamics in oxygen-induced changes in S-layer protein synthesis from *Bacillus stearothermophilus* PV72 and the S-layer-deficient variant T5 in continuous culture and studies of the cell wall composition. *J Bacteriol* **178**, 2108–2117.
- Tailliez, P., Girard, H., Millet, J. & Béguin, P. (1989). Enhanced cellulose fermentation by an asporogenous and ethanol-tolerant mutant of *Clostridium thermocellum*. *Appl Environ Microbiol* **55**, 207–211.
- Tsuboi, A., Uchihi, R., Tabata, R., Takahashi, Y., Hashiba, H., Sasaki, T., Yamagata, H., Tsukagoshi, N. & Udaka, S. (1986). Characterization of the genes coding for two major cell wall proteins from protein-producing *Bacillus brevis* 47: complete nucleotide sequence of the outer wall protein gene. *J Bacteriol* **168**, 365–373.
- Villarejo, M. R. & Zabin, I. (1974). β -Galactosidase from termination and deletion mutant strains. *J Bacteriol* **120**, 466–474.

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