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A Conserved Motif in S-Layer Proteins Is Involved in Peptidoglycan Binding in *Thermus thermophilus*

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There is experimental evidence to suggest that the 100-kDa S-layer protein from *Thermus thermophilus* HB8 binds to the peptidoglycan cell wall. This property could be related to the presence of a region (SLH) of homology with other S-layer proteins and extracellular enzymes (A. Lupas, H. Engelhardt, J. Peters, U. Santarius, S. Volker, and W. Baumeister, J. Bacteriol. 176:1224–1233, 1994). By using specific monoclonal antibodies, we show that similar regions are present in different members of the *Deinococcus-Thermus* phylogenetic group. To analyze the role that the SLH domain plays in vivo and in vitro in *T. thermophilus*, we have obtained a mutant form (*slpA.X*) of the S-layer gene (*slpA*) in which the SLH domain was deleted. The *slpA.X* gene was inserted into the chromosome of the thermophile by gene replacement, resulting in a mutant which expressed a major membrane protein with the size expected from the construction (90 kDa). This protein was identified as the product of *slpA.X* by its differential reaction with monoclonal antibodies. Mutants expressing the SlpA.X protein grow as groups of cells, surrounded by a common external envelope of trigonal symmetry that contains the SlpA.X protein as a main component, thus showing the inability of the SLH-defective protein to attach to the underlying material in vivo. In addition, averaged images of SlpA.X-rich fractions showed a regular arrangement, identical to that built up by the wild-type (SlpA) protein in the absence of peptidoglycan. Finally, we demonstrate by Western blotting (immunoblotting) the direct role of the SLH domain in the binding of the S-layer of *T. thermophilus* HB8 to the peptidoglycan layer.

Paracrystalline surface layers (S-layers) are commonly found within prokaryotes belonging to different phylogenetic groups (22). As the outermost envelope, the functions of S-layers are related to their interaction with the specific environment in which these organisms grow. However, their constant presence in bacteria belonging to the oldest phylogenetic groups and the severe defects shown by S-layer-defective mutants in these groups (13, 17) suggest that primitive S-layers could have played a role essentially related to the maintenance of the cell morphology and envelope integrity (2, 22).

Three-dimensional reconstruction of S-layers has revealed features common among them (22), independently of differences in their specific functions. Essentially, S-layers present a smooth surface which faces outside and a more corrugated one that binds them to the underlying envelope, whatever its nature (peptidoglycan, outer membrane, or lipopolysaccharide). At higher resolution, this corrugated side shows up as wide columns, located at the main symmetry axis, that are interconnected through a network of thin contacts at the surface. This network of contacts generates the smooth face of the S-layer.

This common morphology of the S-layer is built up by a single protein component that is folded in at least two clear domains: a heavy domain, which interacts with other equivalent domains to form the wide columns that bind the S-layer to the cell, and one or more light domains to connect them at the surface (28). However, the resolution of the three-dimensional models available at present does not allow a correlation between structural topology and protein sequence to be made. In addition, the scarcity of both S-layer models and protein sequences and the high divergence found within the latter do not in general allow association of specific sequence motifs or patterns with specific structural features. However, in a recent article, Lupas et al. (20) have described the existence of one or more copies of a protein domain known as the S-layer homology (SLH) region in a number of S-layers and regular membrane proteins from unrelated bacteria. On the basis of the homology, also found with extracellular enzymes from gram-positive bacteria (20), a peptidoglycan-binding function was tentatively assigned to this domain.

Despite belonging to a phylogenetic group different from that of gram positive bacteria, the 100-kDa S-layer protein from *Thermus thermophilus* HB8 was included among those that contain an SLH domain (11, 20). In this 917-amino-acid-long protein (SlpA), the SLH domain was found from amino acid positions 28 to 87, thus suggesting the possibility of an interaction in vivo between the S-layer and the peptidoglycan. Two biochemical data can be argued to support the existence of such interactions. First, the solubilization of SlpA with neutral detergents requires a previous treatment of the envelopes with lysozyme (9) and, second, the hexagonal arrangements of SlpA can be transformed in vitro into trigonal structures through peptidoglycan digestion and Triton X-100 incubation (5). However, the presence of a thick amorphous layer under the S-layer (5) made doubtful the existence of such interactions in vivo.

In this study, we take advantage of both the availability of a thermostable kanamycin resistance selectable marker and the natural competence of *Thermus* cells to replace the wild-type S-layer protein by a mutant protein from which the SLH motif was deleted. We show here that the defective S-layer protein is unable to attach to the cell surface and to form hexagonal arrangements in vivo. In addition, we demonstrate in vitro the direct responsibility of the SLH domain in the binding of the S-layer to the peptidoglycan in *T. thermophilus.*

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MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and growth conditions. *T. thermophilus* HB8 (ATCC 27634) and *Thermus* sp. (ATCC 27737) were obtained from the American Type Culture Collection (Rockville, Md.). *Deinococcus radiodurans* (29), *Thermus aquaticus* YT1 (ATCC 25105), and *T. thermophilus* HB27 were generously given by W. Baumeister, M. Bothe, and Y. Koyama, respectively. The construction of the strain *T. thermophilus* HB27C8 has been described previously (13). *Escherichia coli* DH5αF′ [F′ *supE44* Δ(*lacZYA-arF*)U169 (Φ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (Bethesda Research Laboratories, Gaithersburg, Md.) and JM109 [K-12 λ$^-$ *supE44* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*Z*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (30) were used as hosts for genetic manipulation of plasmids.

T. thermophilus strains were grown at 70°C under strong aeration in a rich medium containing 8 g of Trypticase (BBL, Cockeysville, Md.), 4 g of yeast extract (Oxoid, Hampshire, England), and 3 g of NaCl per liter of tap water, adjusted to pH 7.5. *T. aquaticus* and *Thermus* sp. were grown under identical conditions in a one-half dilution of the same medium. For petri plates, 1.5% (wt/vol) agar was added to solidify the medium. When necessary, 30 μg of kanamycin per ml was added to the plates. The plates were incubated at 70°C in a water-saturated atmosphere.

The *E. coli* strains were grown at 37°C with aeration in M9 minimal medium (23), supplemented with the specific requirements, or in LB medium (19). When a plasmid was present, selective antibiotic was added at 100 μg/ml for ampicillin or 30 μg/ml for kanamycin. Cells were made competent as described elsewhere (8). *D. radiodurans* was grown under aeration at 30°C in LB medium.

Plasmids pPS1 (18), pKKB (13), and pRCS1.BBΔS (13) were previously described.

Transformation of *T. thermophilus*. For plasmid transformation, essentially the method described by Koyama et al. was used (15). *T. thermophilus* HB27C8 was grown at 70°C in a transformation medium as described elsewhere (13).

DNA analysis. Most DNA techniques were carried out as described elsewhere (26). Plasmid DNA was purified from *E. coli* by the alkaline lysis method, and total DNA was purified by the method described by Marmur (21). Restriction enzymes and ligation reactions were performed as described by the manufacturer (Boehringer-Mannheim GmbH, Penzberg, Germany). DNA sequencing was performed by the method described by Sanger et al. (27), modified T7 DNA polymerase (Sequenase 2.0; U.S. Biochemicals), α-^{35}S-dATP (1,000 Ci/mmol) (Amersham), and specific oligonucleotides synthesized by Isogen Bioscience (Maarseen, Holland).

Southern blot analysis of total DNA was performed as described elsewhere (26), with 5 μg per *Bam*HI-digested sample. Hybridization was carried out with the following probes: a 0.8-kbp *Nde*I-*Sma*I fragment purified from plasmid pKT1 (17) for the *kat* (kanamycin adenyltransferase [Kan^r]) gene and a 0.5-kbp *Xho*I-*Pst*I fragment purified from plasmid pMF4 (10) for the *slpA* gene. Uniformly ^{32}P-labeled DNA probes were obtained by using random hexanucleotide primers (Pharmacia), [α-^{32}P]dCTP (3,000 Ci/mmol) (Amersham), and the Klenow fragment. Free nucleotides were removed by Sephadex G-50 column chromatography.

Protein analysis. Cell envelopes were purified (5) and routinely stored at −20°C in 10 mM Tris-HCl (pH 7.8) buffer, at a concentration of approximately 30 mg of protein per ml. Cell envelopes batches were thawed at room temperature immediately before use. Total proteins from these fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16).

Western blotting (immunoblotting). Immunodetection of S-layer proteins from different bacterial strains was performed with the ECL Western blotting analysis system (Amersham International) after electrotransference to an Immobilon P paper (Millipore Ibérica, Madrid, Spain). Polyclonal rabbit antiserum (10) and purified mouse monoclonal antibodies (MAbs) against the S-layer protein from *T. thermophilus* HB8 (7) were used to reveal *slpA* products. The regions to which each MAb recognized the corresponding epitope (7) were 1AE1, between positions 1 and 98; 3EB9, between positions 99 and 196; 2AF1, between positions 197 and 261; 3EB10, between positions 288 and ~450; 2DD11, between this position and 556; and 2BH7, between positions 556 and 728.

Peptidoglycan-binding assays. Total proteins from the *T. thermophilus* strains HB27C8 and HB27CSlpA.X-1 were obtained from sonicated cells (0.5-s pulses for 1 min, maximum power in a Labsonic; B. Brawn) by incubation for 2 min at 92°C in a buffer containing 70 mM Tris-ClH, 2% SDS, and 5 mM EDTA (pH 6.8). Insoluble material was eliminated by centrifugation (15,000 × *g*, 15 min), and total proteins were separated in an SDS–10% PAGE gel at 50 mA of constant current for 16 h. The proteins were then electrotransferred to a nitrocellulose membrane (NC 45; SERVA, Heidelberg, Germany) in a submerged electroblotting system for 6 h at 400 mA in a buffer containing 50 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol. The filter was incubated for 16 h at 4°C in a renaturation buffer containing 20 mM Tris-ClH, 150 mM NaCl, 2.5 mM dithiothreitol, 2.5% (vol/vol) Nonidet P-40, 10% (vol/vol) glycerol, and 5% skim milk (pH 7.5) with three buffer changes and washed in binding buffer (10 mM Tris-ClH, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% [vol/vol] glycerol, and 0.125% [wt/vol] skim milk [pH 8]). The membrane was incubated at 25°C for 3 h in binding buffer containing a sonicated peptidoglycan fraction (24 μg/ml in Ornitin equivalents) from *T. thermophilus* HB8 (24) and was washed again in the same buffer. Peptidoglycan bound to proteins was detected as described above with a specific anti-peptidoglycan rabbit antiserum generously given by H. Schwarz.

Transmission electron microscopy. Bacterial cells were negatively stained with 2% (wt/vol) uranyl acetate after adhesion to carbon-coated grids. Regular arrangements from SlpA and SlpA.X crystals were obtained from whole-cell envelope fractions by treatments with Triton X-100 and EDTA (5), but with higher centrifugation speeds (20,000 × *g*, 15 min). Antigenic determinants in the S-layer were identified by immunoelectron microscopy as previously described (25). Samples were negatively stained with 2% (wt/vol) uranyl acetate. Electron micrographs were recorded in a JEOL 1200EX electron microscope.

Image processing. Electron micrographs were selected by optical diffraction. Digitization of the regular arrays was carried out in an Eikonix charge-coupled device (Applied Imaging), with a pixel size corresponding to 0.45 nm in the specimen plane. The areas (512 by 512 pixels) were processed by Fourier-based methods as previously described (5). The images were averaged without imposing any symmetry. Relevant spots extended to a resolution of as much as 2.5 nm.

RESULTS

The existence of a region conserved among *Thermus* spp. and *D. radiodurans* is revealed by a specific MAb. The availability of a collection of MAbs against different regions of the S-layer protein (SlpA) from *T. thermophilus* HB8 allowed us to analyze the existence of conserved domains within the S-layer proteins of different isolates belonging to the *Thermus-Deinococcus* phylogenetic group (14). For this purpose, we purified cell envelope fractions from *T. thermophilus* HB8, *T. thermophilus* HB27, *Thermus* sp. (ATCC 27737), *T. aquaticus* YT1 (ATCC 25104), and *D. radiodurans* and assayed the presence among them of proteins recognized by polyclonal rabbit antiserum against SlpA. The results are presented in Fig. 1A. The rabbit polyclonal antiserum revealed the presence of proteins immunologically related to SlpA in all of the strains assayed. From these, the 95-, 100-, and 110-kDa proteins detected in *T. thermophilus* HB27, *Thermus* sp., and *D. radiodurans*, respectively, were in accordance with the sizes of their respective S-layer proteins (1, 4, 13). The smaller bands detected in these isolates most probably corresponded to proteolytic fragments of the full-size S-layer protein (1, 13).

A 74-kDa protein was identified in *T. aquaticus*, thus suggesting the existence of an S-layer component also in this strain. In addition, the bands with slower mobility that were detected with the polyclonal antiserum were calcium-dependent aggregates of the S-layer protein, in a way similar to that of the S-layer protein from *T. thermophilus* HB8 (3).

When MAbs against different SlpA regions were used to identify these proteins, only MAb 1AE1 was able to react against them in all of the isolates (Fig. 1B). By contrast, the detection of S-layer bands with other MAbs depended on the strain. While all detected the 95-kDa S-layer protein from the closely related strain *T. thermophilus* HB27, only MAb 3EB10 also recognized the 70-kDa band from *T. aquaticus*, however, none of them was able to identify any protein in *D. radiodurans.*

The results shown in Fig. 1 clearly demonstrated the existence of an epitope located between amino acid positions 1 and 98 (recognized by MAb 1AE1) in the S-layer protein of *T. thermophilus* HB8 that is conserved among different S-layers from the *Deinococcus-Thermus* group. These data are in good agreement with those of sequence comparisons that revealed the existence in this region of a conserved sequence, called the SLH sequence, that was proposed to anchor the S-layer to the peptidoglycan (20).

Deletion of the conserved region in the SlpA protein. To analyze the role that the SLH domain could play in structure and/or attachment of SlpA to the cell, we obtained a mutant form of SlpA in vitro, in which this region was deleted. The process, summarized in Fig. 2, started with the cloning of a 3.6-kbp *Xho*I-*Hin*dIII fragment from plasmid pRCS1.BBΔS in

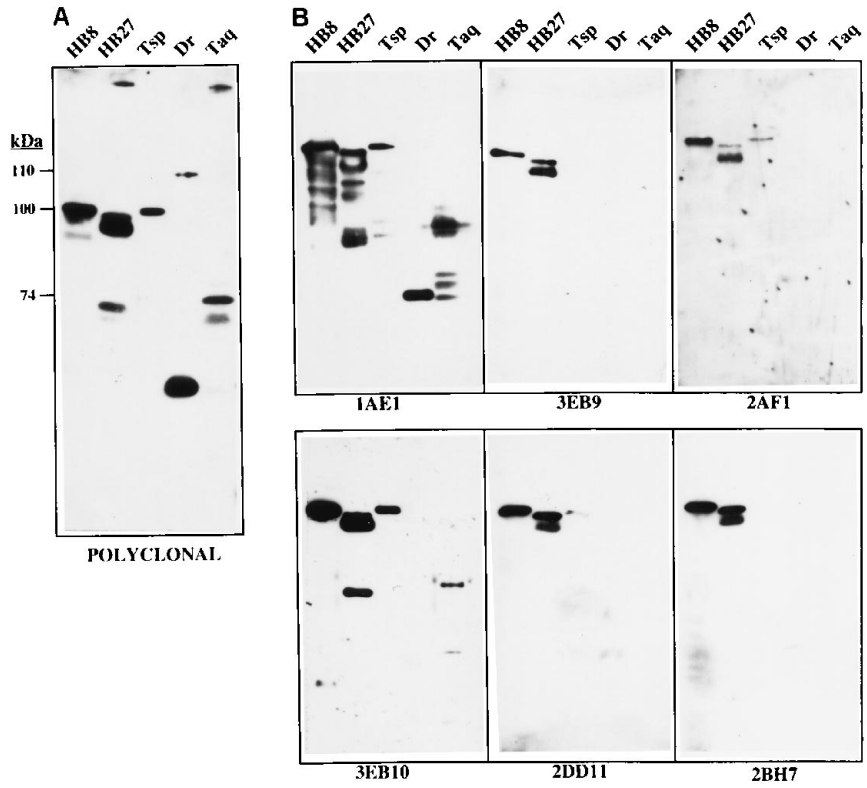

FIG. 1. Epitope conservation among S-layers of the *Thermus-Deinococcus* group. Total proteins from envelope fractions of *T. thermophilus* HB8 (HB8) and HB27 (HB27), *Thermus* sp. ATCC 27737 (Tsp), *D. radiodurans* (Dr), and *T. aquaticus* (Taq), were separated by SDS-PAGE and subjected to Western blotting with rabbit polyclonal antiserum against SlpA (A) and the MAbs against SlpA whose names are labeled at the bottom of the gels (B).

the *Sal*I-*Hin*dIII sites of plasmid pPS1, which contained the signal peptide region of SlpA (from amino acid positions 1 to 34). The plasmid obtained, pPS1.X, contained a mutated form of SlpA in which, as shown at the bottom of Fig. 2, the region from amino acids 34 to 98 was deleted and was replaced by five amino acids included during the construction. This mutated S-layer gene form was called *slpA.X.*

To replace the *slpA* gene of *T. thermophilus* by this construction, we added to the 5′ end of the *slpA.X* gene a 4.8-kbp *Eco*RI fragment from plasmid pKKB (Fig. 2) that contained a thermostable kanamycin resistance gene and sequences upstream of *slpA* (the *glmS* gene, coding for glucosamine-6-P synthase) (12). This plasmid, called pslpA.X, was linearized with *Hin*dIII and was used to transform the strain *T. thermophilus* HB27C8, which is a strain of high transformation efficiency that contains the *slpA* gene from *T. thermophilus* HB8 (13). The kanamycin-resistant colonies that appeared after 48 h of incubation at 70°C were assayed directly by SDS-PAGE for the appearance of a 90-kDa protein, the size expected for the product of *slpA.X*. One of 12 colonies assayed expressed a 90-kDa protein as a major protein and was thus called *T. thermophilus* HB27CSlpA.X-1 and subjected to further analysis.

Expression of *slpA.X.* To determine whether the mutated *slpA.X* gene was present in the chromosome of *T. thermophilus* HB27CSlpA.X-1, total DNA from this strain and from the parental strains (*T. thermophilus* HB27C8) was purified, digested with *Bam*HI, and subjected to Southern blotting. As shown in Fig. 3A, the use of an *slpA* probe revealed the expected 4.8-kbp band in wild-type *T. thermophilus* HB27C8 (lane 1) and the 4.4-kbp band in *T. thermophilus* HB27CSlpA.X-1 (lane 2). When the same blot was hybridized with a *kat* probe (Fig. 3B), a 1.3-kbp band was revealed specifically in the *T. thermophilus* HB27CSlpA.X-1 strain, thus confirming the presence of the *kat* gene. These results demonstrated that the expected gene replacement took place in *T. thermophilus* HB27CSlpA.X-1.

Once we demonstrated that the *slpA.X* gene was inserted into the chromosome, we used specific MAbs to probe whether the 90-kDa protein expressed in the strain *T. thermophilus* HB27CSlpA.X-1 was the product of *slpA.X*. In Fig. 4A, we show a Coomassie blue-stained SDS-PAGE gel in which the 100-kDa wild-type SlpA (lane 1) and the 90-kDa putative SlpA.X (lane 2) proteins were clearly detected. A parallel Western blot with MAb 1AE1 (Fig. 4B) clearly identified the SlpA protein, but not the SlpA.X protein, thus showing the absence of the corresponding epitope in this protein. Concomitantly, the use of MAb 3EB9, which recognizes a region (positions 99 to 196) conserved in both the *slpA* and *slpA.X* genes, allowed the identification of both proteins (Fig. 4C). These results clearly identified the 90-kDa protein expressed in the strain *T. thermophilus* HB27CSlpA.X-1 as the product of the *slpA.X* gene.

Phenotypic analysis of *T. thermophilus* HB27CSlpA.X-1. Electron microscopy images of *T. thermophilus* HB27CSlpA.X-1 revealed the presence of groups of cells surrounded by a common external envelope (Fig. 5B), a defect similar to that described for mutants carrying a complete deletion of the *slpA* gene (13). Nevertheless, in *T. thermophilus* HB27Δ*slpA* mutants, the common external envelope stained poorly and did not present any regular pattern (13), while in *T. thermophilus*

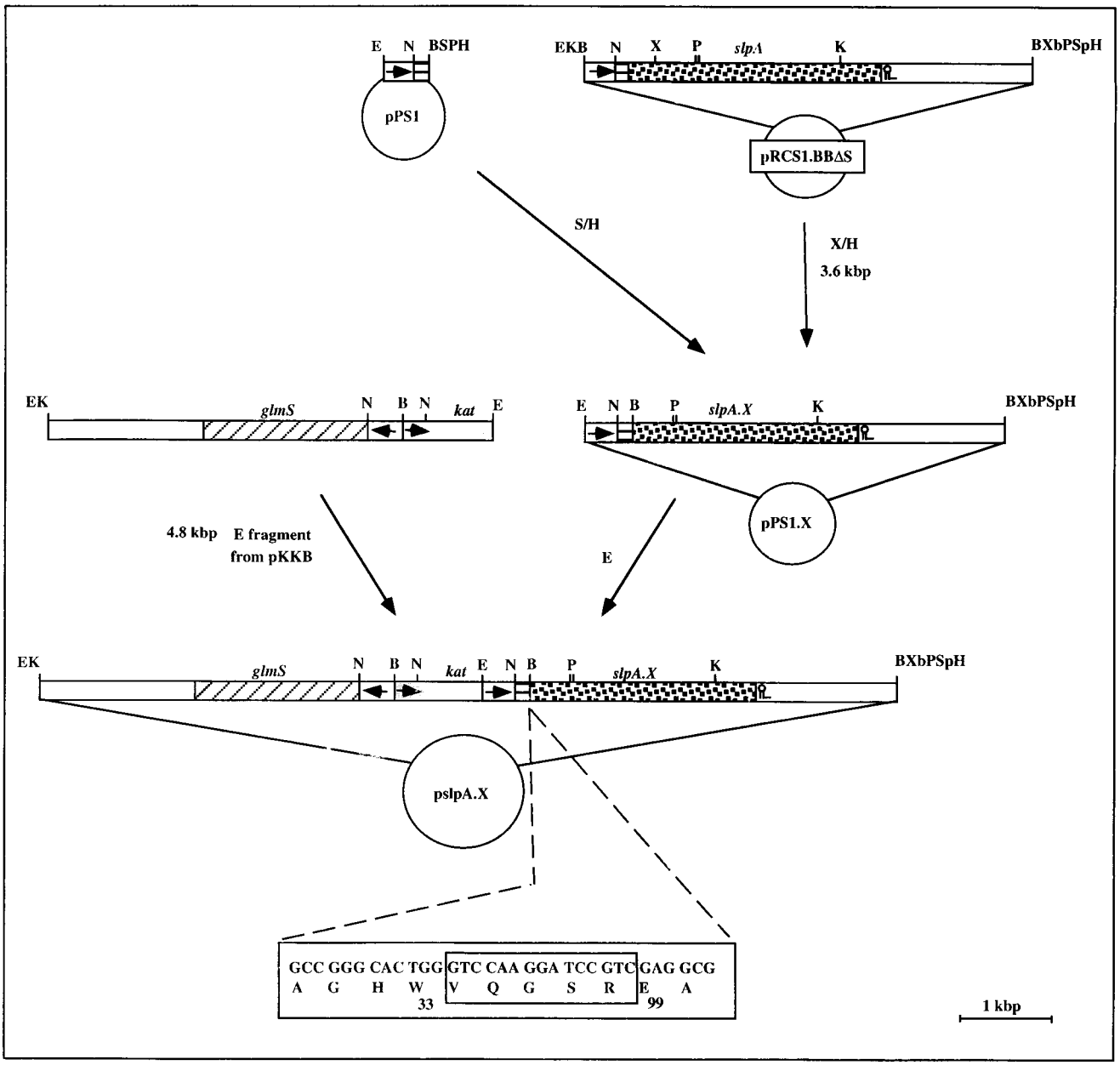

FIG. 2. Construction of the pslpA.X plasmid. The figure represents the procedure through which the pslpA.X plasmid was constructed (see the text for details). Black squares, sequences corresponding to the *slpA* gene sequence; bars, sequences of the 5′ adjacent *glmS* gene. The fusion sequences between positions 33 (W) and 99 (E) of SlpA are framed at the bottom. Restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nde*I; P, *Pst*I; Sp, *Sph*I; Xb, *Xba*I.

HB27CSlpA.X-1, this envelope was easily stained and showed a regular pattern (Fig. 6).

This phenotype could be a consequence of the inability of the SlpA.X protein to attach to the underlying material. However, to support such a hypothesis, we should demonstrate that SlpA.X is actually the main component of this envelope. To probe this, we assayed the detection of its structural component by immunoelectron microscopy with the MAbs used in Fig. 4B and C (1AE1 and 3EB9, respectively) directly on overnight cultures of *T. thermophilus* HB27CSlpA.X-1. As is shown in Fig. 6A, MAb 1AE1 did not recognize this envelope (Fig. 6A), while MAb 3EB9 labeled it completely (Fig. 6B). Thus, these results demonstrated that SlpA.X is a main component of the common external envelope of *T. thermophilus* HB27CSlpA.X-1.

Image analysis of the regular arrangements built up by SlpA.X. To know if the SlpA.X protein was still able to form regular arrays as its parental SlpA protein did, we purified total-cell envelopes from the strain *T. thermophilus* HB27 CSlpA.X-1. This envelope fraction was subjected to the standard protocol for the isolation of hexagonal S-layer arrays in the parental strain (5). The final insoluble fraction, in which the SlpA.X protein was the main component (data not shown), was analyzed by electron microscopy (Fig. 7A), and the images were subjected to Fourier-based processing procedures.

Figure 7B shows the averaged image of the array. The unit

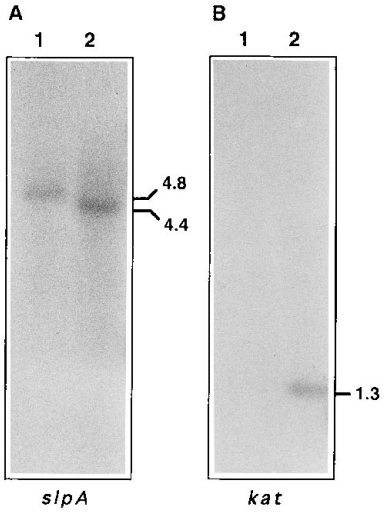

FIG. 3. Southern blot analysis of *T. thermophilus* HB27CSlpA.X-1. Total DNAs (5 μg) from *T. thermophilus* HB27C8 (lanes 1) and *T. thermophilus* HB27CSlpA.X-1 (lanes 2) were digested with *Bam*HI and subjected to Southern blotting. Specific bands, whose sizes (in kilobase pairs) are labeled at the right, were revealed with ^{32}P-labeled probes for the *slpA* (A) and *kat* (B) genes.

cell dimensions (a = b = 18 nm), the overall aspect (a trigonal arrangement), and the detailed morphology (deeply stained channel triplets around apparent threefold centers) are identical to those described for the pS2 arrays build up by the purified SlpA protein (5, 6). These structural similarities between SlpA.X arrays and the arrangements built up by the entire SlpA protein in the absence of peptidoglycan demonstrated that the SlpA.X protein contains the domains needed to produce the intersubunit contacts to build this regular array. Furthermore, they also suggest that the absence of either the peptidoglycan or the sequences deleted in the SlpA.X protein leads to the same morphology, thus supporting the idea that these sequences are involved in peptidoglycan binding in the wild-type bacteria.

The SLH domain is involved in peptidoglycan binding. The above-mentioned results demonstrated that the SlpA.X protein was unable to bind to the underlying cell layer in vivo. To see whether this was due to the absence of a peptidoglycan-binding motif contained in the SLH region, we developed a method to assay the direct binding of peptidoglycan fragments to membrane proteins (Fig. 8). For this purpose, we separated total proteins from *T. thermophilus* HB27C8 (lanes 1) and HB27CSlpA.X (lanes 2) by SDS-PAGE and transferred them to a nitrocellulose filter. The proteins were then subjected to a renaturation process and incubated with sonication-generated fragments of a crude peptidoglycan fraction from *T. thermophilus* HB8 that were further detected with an antiserum against peptidoglycan. As is shown in Fig. 8B, a band corresponding to the wild-type SlpA protein was strongly labeled, while the SlpA.X protein remained undetected. In addition, we also could detect other labeled bands in this strain which most probably corresponded to degradation products and oligomers of SlpA, as has been previously shown (7, 9, 10). As a negative control of the antiserum specificity, a parallel Western blot was carried out in which the peptidoglycan fraction was not added, resulting in the complete absence of labeling after incubation with the antiserum (data not shown).

DISCUSSION

In a recent article, Lupas et al. (20) have proposed the existence of a conserved region within S-layers from gram-positive organisms whose function could be related to the attachment of the regular array to the peptidoglycan cell wall. In the S-layer protein of *T. thermophilus* HB8 (SlpA), there is a single copy of such a conserved motif, starting at position 24 and ending at position 87. The availability of a MAb (1AE1) specific for an epitope located in this region (7) allowed us to demonstrate the existence of similar sequences in the S-layer proteins from phylogenetically related isolates, including *D. radiodurans* (Fig. 1), thus supporting the actual existence and an even wider extension of the homology proposed by Lupas et al. (20).

To demonstrate in vivo the possible cell attachment role of the SLH domain of SlpA, we constructed a mutant form of its coding gene in which this region was deleted from amino acids 34 (to keep the whole signal peptide) to 98 (Fig. 2). The mutant gene, *slpA.X*, was used to replace the wild-type gene by using an adjacent kanamycin resistance gene and regions upstream of *slpA* to force and direct a double recombination process. For this transformation, we used the high-transformation-efficiency strain *T. thermophilus* HB27C8, which carries the *slpA* gene from the strain *T. thermophilus* HB8.

In the kanamycin-resistant strain subsequently selected, which was called *T. thermophilus* HB27CSlpA.X-1, a Southern blot experiment demonstrated the expected gene replacement through the identification of specific *Bam*HI chromosomic fragments with *slpA* and *kat* probes (Fig. 3). This strain expressed a protein whose mobility (90 kDa) was similar to that expected from the sequence of the *slpA.X* gene (89.5 kDa). Its identification as the product of *slpA.X* was unequivocally demonstrated by the absence of labeling with MAb 1AE1, which is specific for the deleted region, while MAb 3EB9, which is specific for the following sequences, strongly labeled this band (Fig. 4).

The microscopic analysis of *T. thermophilus* HB27CSlpA.X-1 revealed defects in morphology somehow similar to those described for *slpA* deletion mutants (13). Especially relevant was a defect in the attachment of an external layer, which leads through internal division to the formation of groups of cells inside a common envelope (Fig. 5B). The nature of this envelope was analyzed by electron immunomicroscopy with MAbs 1AE1 and 3EB9. Again, the results were clear. While MAb 1AE1 was unable to label the envelope, MAb 3EB9 showed a

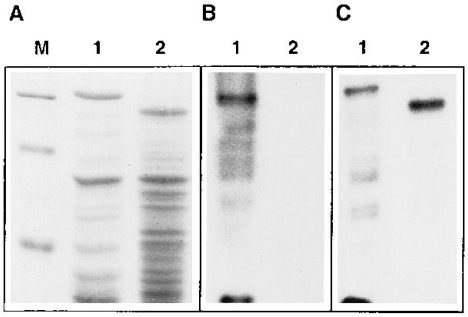

FIG. 4. Identification of the expression product of *slpA.X*. (A) Coomassie blue staining of total envelope proteins from *T. thermophilus* HB27C8 (lane 1) and *T. thermophilus* HB27CSlpA.X-1 (lane 2); (B) parallel Western blotting with MAb 1AE1; (C) parallel Western blotting with MAb 3EB9. Sample M in panel A contains size markers of 97.4, 66.2, and 45 kDa, respectively.

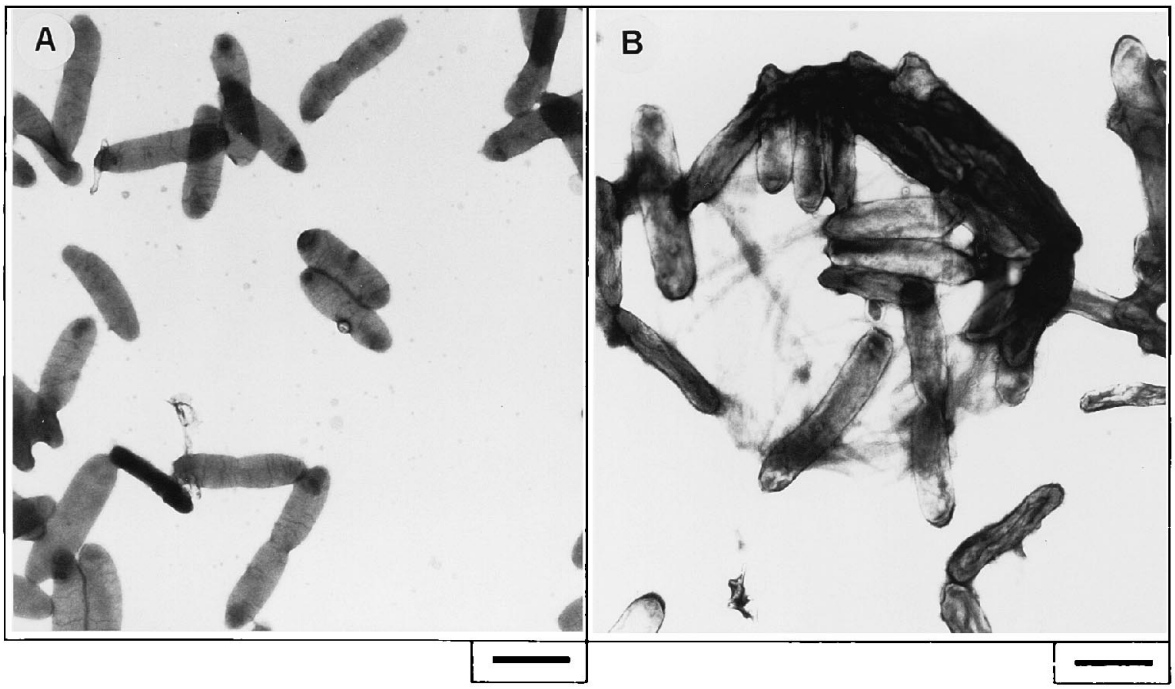

FIG. 5. Microscopic analysis of *T. thermophilus* HB27CSlpA.X-1. Overnight cultures of *T. thermophilus* HB27C8 (A) and *T. thermophilus* HB27CSlpA.X-1 (B) were stained with uranyl acetate on carbon-coated grids and observed by transmission electron microscopy. Bars, 1 μm.

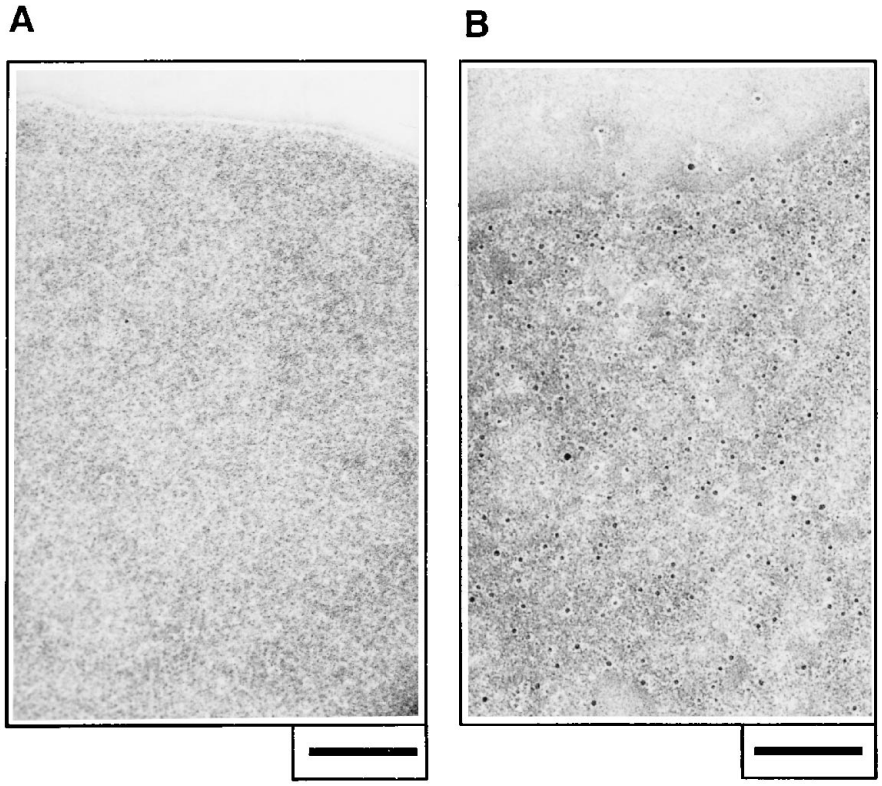

FIG. 6. Identification of SlpA.X as a component of the common external envelope in *T. thermophilus* HB27CSlpA.X-1. Cells from an overnight culture of *T. thermophilus* HB27CSlpA.X-1 were adsorbed to ionized carbon-coated grids and were immunolabeled with MAb 1AE1 (A) or 3EB9 (B). Bars, 100 nm.

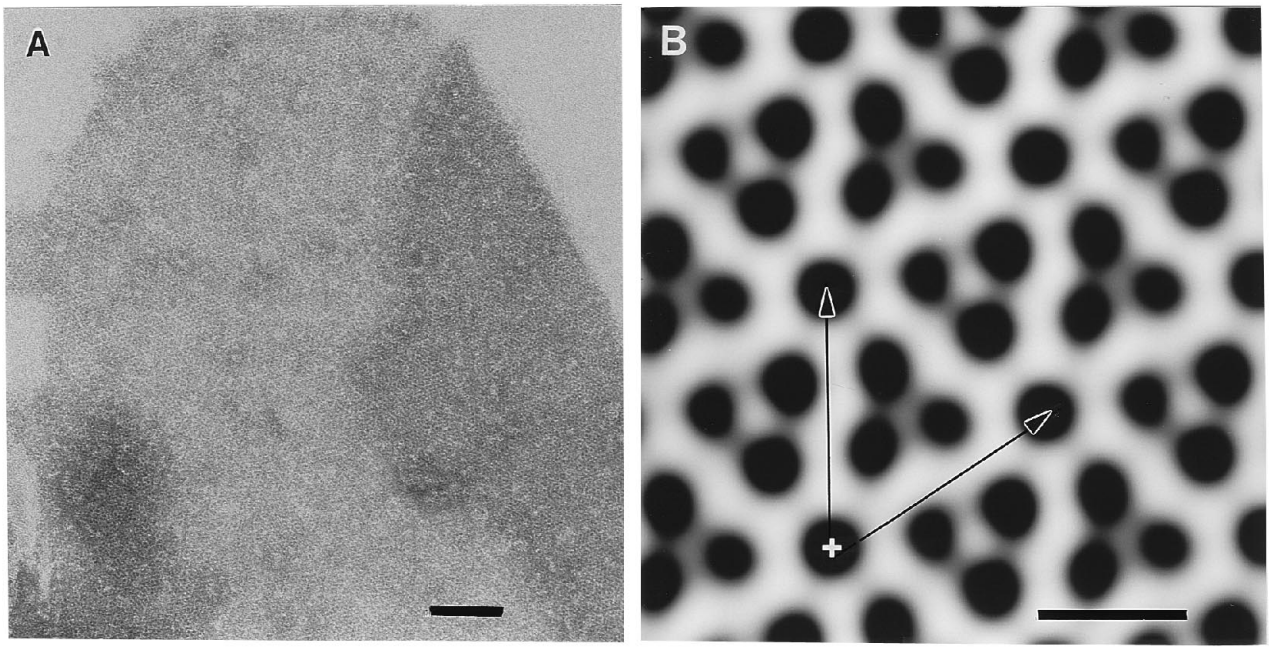

FIG. 7. Averaging of two-dimensional arrays of SlpA.X protein. (A) Negatively stained SlpA.X crystalline sheet purified from cell envelopes. The right side shows a fold of the sheet over itself, depicting the typical moiré pattern from superposition of crystalline areas. Bar, 100 nm. (B) Fourier-averaged image from a well-preserved area (230 by 230 nm^2). No symmetry was imposed during the processing. The arrows mark the unit cell vectors. Bar, 10 nm.

clear detection of the corresponding epitope (Fig. 6). Thus, these data demonstrated that the SlpA.X protein was a main component of the common external envelope of *T. thermophilus* HB27CSlpA.X-1 and, consequently, that the absence of the deleted SLH region made the SlpA.X protein unable to bind to the underlying layer in vivo.

A preliminary biochemical study of SlpA.X demonstrated a solubility in Triton X-100 higher than that of SlpA, thus requiring higher centrifugation speed and time to recover the protein in the insoluble fraction (data not shown). By adjusting the purification conditions, it was possible to obtain an SlpA.X-rich insoluble fraction after an extraction procedure similar to that followed to obtain hexagonal arrangements of the SlpA protein (5). In contrast to what happened with the wild-type SlpA protein, we never detected in these fractions the characteristic S-layer hexagonal array with the SlpA.X protein despite the presence of peptidoglycan. In fact, the regular arrangements detected had a trigonal symmetry (Fig. 7A), and the bidimensional image reconstruction (Fig. 7B) revealed a geometry and unit cell vectors indistinguishable from those of the arrangements built up by the wild-type SlpA protein in the absence of peptidoglycan (5).

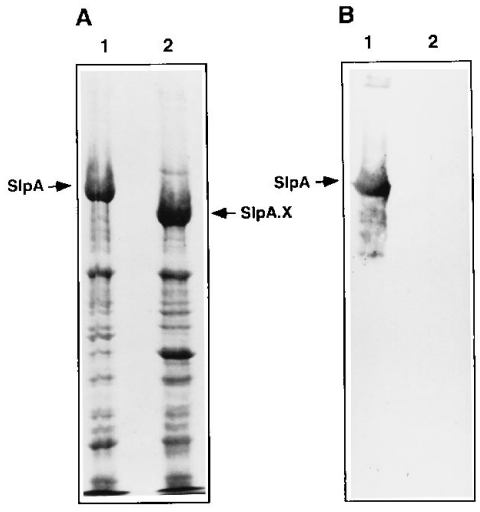

FIG. 8. Binding of peptidoglycan to the SlpA protein. (A) Coomassie blue staining of total cell proteins (300 μg) from *T. thermophilus* HB27C8 (lane 1) and HB27CSlpA.X (lane 2); (B) parallel Western blot showing the binding of peptidoglycan fragments to identical amounts of total proteins from *T. thermophilus* HB27C8 (lane 1) and HB27CSlpA.X (lane 2).

The inability of the SlpA.X protein to attach to the cell surface and to form hexagonal arrays and its rearrangement in layers indistinguishable from those built up by the SlpA protein in the absence of peptidoglycan strongly supported the hypothesis that the SLH domain of the S-layer protein of *T. thermophilus* HB8 was responsible for the attachment to the peptidoglycan cell wall. To demonstrate this, we took advantage of the availability of an antiserum against the peptidoglycan layer to detect its binding to membrane proteins renaturalized on nitrocellulose filters (Fig. 8). By this method, we could detect a strong labeling of the SlpA protein in the lanes (lanes 1) containing total proteins of *T. thermophilus* HB27C8. The complete absence of labeling in the lane corresponding to total proteins of *T. thermophilus* HB27CSlpA.X (lanes 2) demonstrated that the deletion of the SLH domain in the SlpA.X protein was responsible for the inability of this protein to bind to the peptidoglycan layer.

The demonstrated implication of the SLH domain in peptidoglycan binding in *T. thermophilus* could explain the conservation of such a homologous region among otherwise highly divergent S-layers from bacterial groups as unrelated as gram-positive bacteria, *Thermotoga maritima* (20), and *Deinococcus-Thermus.*

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