JOURNAL OF BACTERIOLOGY, Oct. 1996, p. 5602–5609 Vol. 178, No. 19
0021-9193/96/$04.00+0

Evidence that an N-Terminal S-Layer Protein Fragment Triggers the Release of a Cell-Associated High-Molecular-Weight Amylase in *Bacillus stearothermophilus* ATCC 12980

EVA M. EGELSEER, INGRID SCHOCHER, UWE B. SLEYTR, AND MARGIT SÁRA*

Zentrum für Ultrastrukturforschung and Ludwig Bolzmann-Institut für Molekulare Nanotechnologie, Universität für Bodenkultur, A-1180 Vienna, Austria

Received 3 May 1996/Accepted 23 July 1996

During growth on starch medium, the S-layer-carrying *Bacillus stearothermophilus* ATCC 12980 and an S-layer-deficient variant each secreted three amylases, with identical molecular weights of 58,000, 122,000, and 184,000, into the culture fluid. Only the high-molecular-weight amylase (hmwA) was also identified as cell associated. Extraction and reassociation experiments showed that the hmwA had a high-level affinity to the peptidoglycan-containing layer and to the S-layer surface, but the interactions with the peptidoglycan-containing layer were stronger than those with the S-layer surface. For the S-layer-deficient variant, no changes in the amount of cell-associated and free hmwA could be observed during growth on starch medium, while for the S-layer-carrying strain, cell association of the hmwA strongly depended on the growth phase of the cells. The maximum amount of cell-associated hmwA was observed 3 h after inoculation, which corresponded to early exponential growth. The steady decrease in cell-associated hmwA during continued growth correlated with the appearance and the increasing intensity of a protein with an apparent molecular weight of 60,000 on sodium dodecyl sulfate gels. This protein had a high-level affinity to the peptidoglycan-containing layer and was identified as an N-terminal S-layer protein fragment which did not result from proteolytic cleavage of the whole S-layer protein but seems to be a truncated copy of the S-layer protein which is coexpressed with the hmwA under certain culture conditions. During growth on starch medium, the N-terminal S-layer protein fragment was integrated into the S-layer lattice, which led to the loss of its regular structure over a wide range and to the loss of amylase binding sites. Results obtained in the present study provide evidence that the N-terminal part of the S-layer protein is responsible for the anchoring of the subunits to the peptidoglycan-containing layer, while the surface-located C-terminal half could function as a binding site for the hmwA.

Crystalline bacterial cell surface layers (S-layers) represent an almost universal feature of the archaeobacterial cell envelopes and have been detected in hundreds of different species of nearly every taxonomic group of walled eubacteria (for reviews, see references 3, 4, 5, 22, and 29–31). The oblique (p1, p2), square (p4), and hexagonal (p3, p6) lattices are formed of assemblies of identical protein or glycoprotein subunits with molecular weights ranging from 30,000 to 200,000.

S-layer-carrying *Bacillus stearothermophilus* strains can produce large amounts of exoenzymes such as amylases and proteases. Although it has been reported for most *Bacillus* strains that exoenzyme secretion occurs in the stationary growth phase in the course of an increased autolytic activity (1, 23), it was recently demonstrated for the S-layer-carrying strain *B. stearothermophilus* DSM 2358 that amylase secretion starts in the early exponential growth phase (7). Since at this stage of bacterial growth the cell surface is continuously extended, exoenzyme secretion can occur at sites of peptidoglycan and S-layer lattice growth, as well as through the pores arranged in the S-layer lattice (7, 27). In this context, it was suggested that S-layers from members of the family *Bacillaceae* could delineate a kind of periplasmic space in cell envelopes of gram-positive organisms and consequently delay or "control" the release of exoenzymes (6, 12, 33).

In many S-layer proteins, three repeats of about 50 amino acids at the N-terminus, which were also detected at the very C-terminal region of several exoenzymes and exoproteins including the pullulanase of *Thermoanaerobacterium thermosulfurigenes* (20, 32) and the outer layer proteins OlpA and OlpB of *Clostridium thermocellum* (10, 18, 24, 25), were detected. Since these exoproteins were identified as being cell surface associated, it was suggested that a so-called S-layer-homologous domain could enhance the capability of proteins to bind permanently or transiently to the peptidoglycan-containing layer (8, 9, 14, 18, 19).

In a previous report, the putative role of the S-layer from *B. stearothermophilus* DSM 2358 as an adhesion site for a high-molecular-weight amylase (hmwA) was confirmed by affinity studies and immunolabeling techniques (7). Binding of the cell-associated amylase of *B. stearothermophilus* DSM 2358 was suggested to occur to protein domains either located at the surface of the crystal lattice or exposed inside the pores, while integration of the enzyme into the S-layer lattice could be excluded (7). The S-layer-associated amylase from *B. stearothermophilus* DSM 2358 showed no affinity to the peptidoglycan-containing layer.

In the present study, the role of the S-layer lattice with regard to exoenzyme adhesion was investigated for the closely related *B. stearothermophilus* ATCC 12980. As described for *B. stearothermophilus* DSM 2358, this organism possesses an oblique S-layer lattice and secretes three amylases into the culture fluid. Comparative studies with its S-layer-deficient variant ATCC 12980-9/1 allowed us to gather further information on the importance of the S-layer lattice for exoenzyme secretion.

* Corresponding author. Mailing address: Zentrum für Ultrastrukturforschung, Universität für Bodenkultur, Gregor-Mendelstr. 33, A-1180 Vienna, Austria. Phone: 0043 1 47654 2208. Fax: 0043 1 34 61 76.

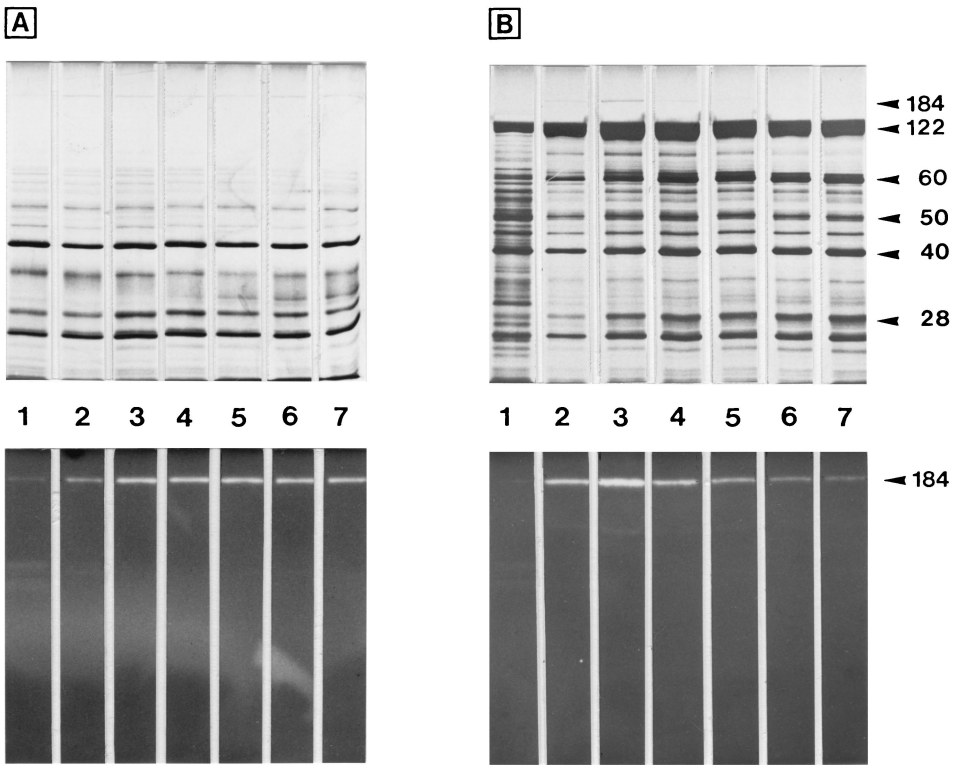

FIG. 1. SDS-PAGE patterns of whole-cell extracts from the S-layer-deficient variant (A) and the S-layer-carrying strain *B. stearothermophilus* ATCC 12980 (B) cultivated on complex medium. Samples were taken 1 to 7 h after inoculation. Protein bands were visualized by silver staining; bands with amylolytic activity could be detected by applying iodine-starch reagent to the SDS gels. Values to the right are molecular weights (in thousands). In the S-layer-deficient variant, the hmwA showed a constant level of cell association (A). In S-layer-carrying cells, high-level expression of a protein with a molecular weight of 60,000 (P_{60}) was accompanied by a reduced level of cell association and the release of the hmwA into the culture fluid (B).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The S-layer-carrying strain *B. stearothermophilus* ATCC 12980 and the S-layer-deficient variant designated 12980-9/1 (21) were grown in 300-ml shaking flasks on 80 ml of complex medium [4.0 g of peptone, 4.0 g of laboratory Lemco powder, 4.0 g of $(NH_4)_2HPO_4$, 1.0 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of soluble starch (Merck) per liter (pH 7.0)] for up to 17 h. For inoculation, a bacterial suspension (3 ml) in the late exponential growth phase, cultivated on SVIII medium (2) without any carbon source, was used. Under these conditions, no amylases were produced. For the surveillance of bacterial growth, the pH and the optical density of the culture at 600 nm (Beckman spectrophotometer, model 25) were determined. Protein bands with amylolytic activity in culture fluids or cell pellets were visualized in situ on sodium dodecyl sulfate (SDS) gels (17) as described by Lacks and Springhorn (16).

Specimen preparation for electron microscopy. Ultrathin sectioning, freeze-etching of whole cells grown on SVIII and complex media, negative staining of S-layer self-assembly products and cell wall fragments, and electron microscopy were performed as previously described (21).

Preparation of cell wall fragments and S-layer self-assembly products, and isolation of peptidoglycan-containing sacculi. Cell wall fragments of whole cells from the S-layer-carrying strain and the S-layer-deficient variant were prepared according to the procedure of Sleytr and Glauert (28), except that whole cells were broken by ultrasonification under the conditions described previously (21). The purity of cell wall fragments was checked by SDS-polyacrylamide gel electrophoresis (PAGE).

For the production of S-layer self-assembly products, the S-layer protein from cell wall fragments from the S-layer-carrying strain was extracted with guanidinium hydrochloride (GHCl) (5 M GHCl in 50 mM Tris-HCl buffer [pH 7.2]) for 2 h at 20°C. After centrifugation at 40,000 × *g* for 20 min at 20°C, the supernatant containing the extracted S-layer protein was dialyzed against distilled water at 4°C for 24 h. S-layer self-assembly products were sedimented by centrifugation of suspensions at 40,000 × *g* for 20 min. Both sedimented S-layer self-assembly products and clear supernatants were checked by SDS-PAGE.

For the isolation of peptidoglycan-containing sacculi, the treatment of cell wall fragments with 5 M GHCl for 2 h at 20°C (as described before) was followed by a second extraction with 5 M GHCl for 20 min at 20°C. Subsequently, the pellet consisting of peptidoglycan-containing sacculi was washed four times with 50 mM Tris-HCl buffer (pH 7.2) at 20°C, suspended in a small volume of distilled water, frozen at −18°C, and lyophilized.

Extraction of hmwA from S-layer-carrying strain and S-layer-deficient variants with different concentrations of GHCl solutions. Cells from the stationary growth phase of the S-layer-carrying and the S-layer-deficient strain cultivated in 80 ml of complex medium were collected by centrifugation at 17 h after inoculation. Subsequently, the cells were washed twice with 50 mM Tris-HCl buffer (pH 7.2) at 20°C. Aliquots of the cell pellets (170 mg) were incubated with 2 or 4 M GHCl in 50 mM Tris-HCl buffer (pH 7.2) at 4°C for 10 min. After centrifugation of the suspensions at 40,000 × *g* at 4°C for 15 min, the remaining cell pellets were washed twice with 50 mM Tris-HCl buffer (pH 7.2) and subjected to SDS-PAGE. Protein bands with amylolytic activity were detected in situ on SDS gels as previously described (16). The pellets of untreated cells obtained after the incubation of cells in 50 mM Tris-HCl buffer (pH 7.2) were taken as a control.

Investigation of interactions between hmwA and isolated cell wall components (peptidoglycan-containing sacculi and S-layer self-assembly products). The hmwA used in this study was extracted with 4 M GHCl in 50 mM Tris-HCl buffer (pH 7.2) for 10 min at 4°C from cell wall fragments of the S-layer-deficient variant. For refolding, the GHCl-extracted amylase was dialyzed against distilled water for 24 h at 4°C. To study the reassociation between the hmwA and cell wall components, lyophilized samples of isolated peptidoglycan-containing sacculi (1 mg) and S-layer self-assembly products (1 mg) were incubated with 1 ml of a solution containing approximately 50 μg of hmwA for 30 min at 20°C. After centrifugation of the suspensions (40,000 × *g*, 20 min, 4°C), the supernatants and washed pellets of peptidoglycan-containing sacculi and S-layer self-assembly products were subjected to SDS-PAGE. Specific binding of amylase was visualized by silver staining and in situ detection of amylase activity (16).

Studies on the affinity between an S-layer-associated protein with a molecular weight of 60,000 (P_{60}), S-layer-carrying cell wall fragments, and peptidoglycan-containing sacculi. For studying the affinity between P_{60} and the S-layer surface, cell wall fragments from cells which were grown on SVIII medium and completely covered with an S-layer lattice on both sides of the peptidoglycan-containing layer were used. Lyophilized S-layer-carrying cell wall fragments (2 mg) were incubated with 1 ml of supernatant of an S-layer self-assembly suspension containing mainly P_{60} (approximately 1 mg/ml of 50 mM Tris-HCl buffer [pH 7.2]) at 20°C for 40 min.

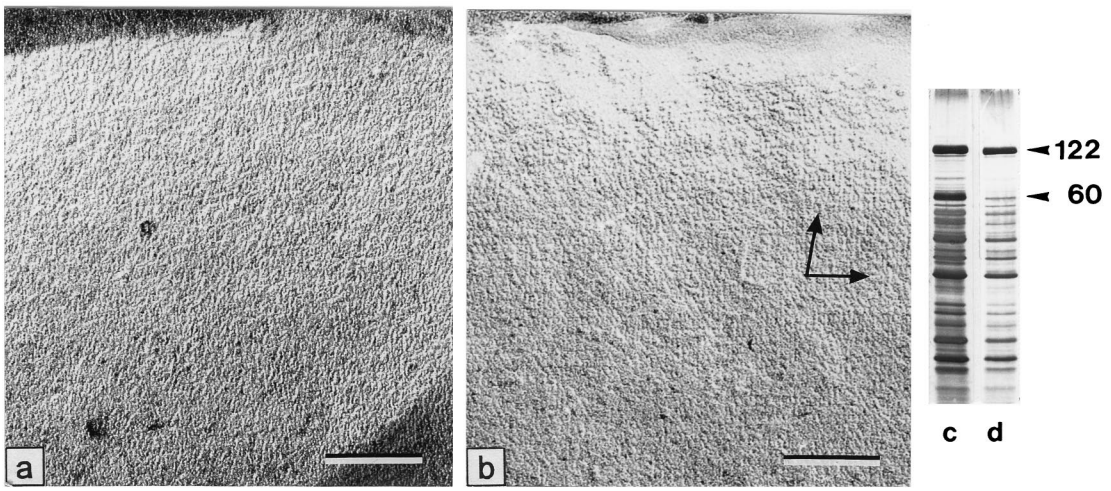

FIG. 2. Freeze-etched preparations (a and b) and SDS-PAGE patterns (c and d) of whole-cell extracts from the S-layer-carrying strain *B. stearothermophilus* ATCC 12980 grown either on complex medium (a and c) or on SVIII medium (b and d). Values to the right are molecular weights (in thousands). Arrows indicate base vectors. Bars, 100 nm.

To investigate whether the disintegration of the S-layer lattice is necessary for the binding of P_{60}, 2 mg of lyophilized S-layer-carrying cell wall fragments was incubated with 1 ml of supernatant of an S-layer self-assembly suspension containing mainly P_{60} (approximately 1 mg/ml) in the presence of 5 M GHCl in 50 mM Tris-HCl buffer (pH 7.2) at 20°C for 40 min. For investigating the affinity of P_{60} to isolated peptidoglycan-containing sacculi, 2 mg of lyophilized peptidoglycan-containing sacculi from the S-layer-carrying strain was incubated with 1 mg of lyophilized S-layer self-assembly suspension containing both P_{60} and the S-layer protein, in comparable amounts, in the presence of 5 M GHCl at 20°C for 30 min. After centrifugation of the suspensions (40,000 × *g*, 4°C, 15 min), GHCl was removed by dialysis for 3 h at 20°C. Subsequently, washed pellets and supernatants were subjected to SDS-PAGE.

Production of antisera against hmwA (M_r 184,000), S-layer protein (M_r 122,000), and P_{60}. The S-layer protein and P_{60} used for immunization were isolated from dialyzed 5 M GHCl extracts prepared from cell wall fragments of the S-layer-carrying strain which contained both proteins. For the isolation of the hmwA, dialyzed GHCl extracts of the S-layer-deficient variant were used. For the production of polyclonal antisera, the proteins in the dialyzed and lyophilyzed GHCl extracts were separated by SDS-PAGE with a separation gel acrylamide concentration of 7.5%. After visualization of the protein bands with Coomassie brilliant blue R 250 (0.1% in distilled water; Merck), gel slices were excised, fragmented, and lyophilized. The protein content of one protein band was estimated densitometrically (Hirschmann Elscript 400 AT/SM densitometer) on silver-stained SDS gels.

Antisera against the three different proteins were produced in rabbits by the subcutaneous injection of samples, each of which contained approximately 30 to 40 μg of protein in 1 ml of 0.9% NaCl solution. Injections were performed three times at 3-week intervals. The antisera were collected from each rabbit 6 weeks after the final injection by bleeding of the ear vein. Complement inactivation in all antisera was achieved by heating at 56°C for 15 min.

SDS-PAGE and immunoblotting. SDS-PAGE was carried out as previously described (17). The immunospecificities of all antisera were investigated by immunoblotting (13). Incubation with antisera was carried out either at a 1:5,000 or 1:10,000 dilution of antisera in 3% bovine serum albumin (Hämosan, ●●●, Austria) in Tris-buffered saline at 37°C for 2 h. Subsequently, blots were incubated with goat anti-rabbit immunoglobulin G (Sigma) at a 1:3,000 dilution for 1 h at 20°C. For the detection of bound alkaline phosphatase-conjugated antiserum, 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) and 4-Nitro Blue Tetrazolium chloride (Boehringer Mannheim) were used as the chromogenic substrate.

Immunogold labeling of hmwA on whole cells of S-layer-carrying and S-layer-deficient variants and electron microscopic examination. Immunogold labeling was accomplished with cells from the early stationary growth phase (4 h after inoculation) which were cultivated in shaking flasks on 80 ml of complex medium containing 1.0 g of soluble starch per liter. Cell pellets from a 2-ml cell suspension were incubated with antiserum against the hmwA at a 1:10 dilution in phosphate-buffered saline for 1 h at 4°C. Washed cells were subsequently treated with 10 μl of a concentrated protein A-colloidal gold conjugate (5 nm; Sigma). After 1 h of incubation at 4°C, unbound protein A-colloidal gold conjugate was removed by three cycles of centrifugation in distilled water. The final cell pellets were suspended in 20 μl of distilled water, and the unfixed and unstained cells were immediately applied to Pioloform-filmed carbon-coated copper grids which had been rendered hydrophilic by glow discharge. Preparations were examined in a Philips CM 100 electron microscope, operated at 80 kV, with a 30-μm-diameter objective aperture.

Peptide mapping of S-layer protein and P_{60}. Endoproteinase Glu-C (*Staphylococcus aureus* V8 protease; Sigma P-6181) was used for proteolytic cleavage of the isolated S-layer protein and P_{60}. For the isolation of both proteins, preparative gel electrophoresis was performed as described for the production of polyclonal antisera. Peptide mapping was carried out by the in-gel system, whereby protein cleavage was accomplished directly in the stacking gel. Excised protein bands were incubated in equilibration solution (0.1% SDS in 0.125 M Tris-HCl buffer [pH 6.8]) for 20 min at 20°C. Subsequently, gel slices were loaded into the sample well of a 4% stacking gel and overlaid with a fresh dilution of endoproteinase Glu-C in enzyme diluent (10% [wt/vol] glycerol, 0.1% SDS, and 0.0001% bromphenol blue in 0.125 M Tris-HCl buffer [pH 6.8]). The protease-to-protein ratio was in the range of 1:60 and 1:40 for the S-layer protein and P_{60}, respectively. For proteolytic digestion, the electrophoresis was stopped when the dye front had reached the middle of the stacking gel and the whole Mini-PROTEAN II slab cell (Bio-Rad Laboratories) was incubated in a water bath for 1 h at 37°C. Following incubation, the resultant fragments were separated on a 12% acrylamide separation gel and visualized by silver staining. The similarity between cleavage products was investigated by the immunoblot technique (13).

N-terminal sequencing of S-layer protein and P_{60}. The S-layer protein and P_{60} (both contained in the dialyzed 5 M-GHCl extracts from cell wall fragments of the S-layer-carrying strain) were blotted to polyvinylidene difluoride membranes (Millipore P^{SQ}; Millipore Corp.). N-terminal sequencing of excised blot bands (~20 nmol of each protein) was performed on a reverse-phase high-pressure liquid chromatography system (Applied Biosystems) equipped with a PTH-RP C_{18} column.

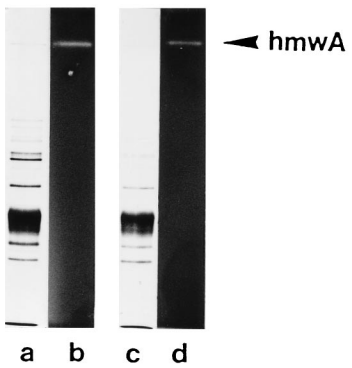

FIG. 3. SDS-PAGE patterns of cell extracts from the S-layer-deficient variant of *B. stearothermophilus* ATCC 12980. As a control, whole cells were incubated in 50 mM Tris-HCl buffer (pH 7.0) (lanes a and b). Whole cells were treated with 2 M GHCl (lanes c and d). Lanes a and c show silver staining results of cell pellets; lanes b and d show results from in situ detection of amylases.

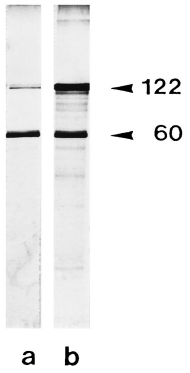

FIG. 4. SDS-PAGE patterns of supernatants (lane a) and sedimented S-layer self-assembly products (lane b) after centrifugation of dialyzed GHCl extracts. Values to the right are molecular weights (in thousands). In supernatants, only small amounts of S-layer protein but at least 50% of the protein with a molecular weight of 60,000 (P_{60}) could be detected while the residual 50% of P_{60} was incorporated into self-assembly products.

Determination of protease and lipase activities. Protease activity of P_{60} was investigated with azocasein as the substrate (34). Lipase activity was determined as described by Gowland et al. (11).

Growth of the S-layer-carrying strain in the presence of protease inhibitors. The S-layer carrying strain *B. stearothermophilus* ATCC 12980 was grown in 100-ml shaking flasks on 25 ml of complex medium at 55°C. A bacterial suspension (1 ml) in the late exponential growth phase cultivated on SVIII medium without any carbon source was used for inoculation.

To study the influence of protease inhibitors on the production of P_{60}, eight different protease inhibitors (protease inhibitors set 1206 893; Biochemica Boehringer Mannheim) were added separately to the bacterial suspensions as follows (concentrations [micrograms per milliliter] are given in parentheses): Prefabloc (100), EDTA (0.2), bestatin (20), leupeptin (0.5), phosphoramindon (120), aprotinin (2), E-64 (10), and antipain-dihydrochloride (50). Aliquots of cell suspensions were taken 1, 2, 3, 4, 5, and 24 h after the addition of protease inhibitors; cell pellets and culture supernatants were investigated by SDS-PAGE.

RESULTS

Comparative studies on growth and amylase production of the S-layer-carrying strain *B. stearothermophilus* ATCC 12980 and the S-layer-deficient variant designated 12980-9/1. The S-layer-deficient variant designated 12980-9/1 (Fig. 1A) was a spontaneous mutant of the S-layer-carrying *B. stearothermophilus* ATCC 12980 (Fig. 1B) (21). During growth on SVIII medium, the S-layer-carrying strain (Fig. 1B) produced an S-layer protein with a molecular weight of 122,000. With the exception of the S-layer protein band, SDS-PAGE patterns from both organisms were identical. No amylase production was observed on SVIII medium.

As determined by SDS-PAGE, by applying the silver staining method and in situ detection of amylase activity, both strains produced three amylases, with molecular weights of 58,000, 122,000, and 184,000, during growth on starch-containing complex medium (Fig. 1, lanes 2 to 7). The enzymes were already secreted into the culture fluid in the early logarithmic growth phase, 2 h after inoculation (data not shown). hmwA was the only enzyme that was found to be cell associated (Fig. 1).

The S-layer-carrying strain and the variant lacking the S-layer revealed significant differences regarding secretion and cell-association of the hmwA. The hmwA produced by the

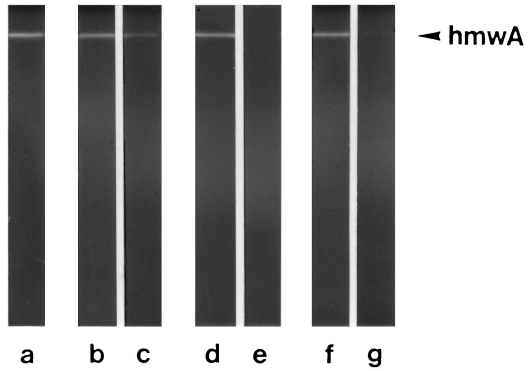

FIG. 5. SDS-PAGE patterns demonstrating the affinity of the hmwA (lane a) to S-layer self-assembly products (lanes b and c) and to isolated peptidoglycan-containing sacculi of the S-layer-carrying strain (lanes d and e) and the S-layer-deficient variant (lanes f and g) of *B. stearothermophilus* ATCC 12980. Lane a shows the soluble hmwA used for affinity studies. Lanes b, d, and f demonstrate binding of the hmwA to S-layer self-assembly products (lane b) and to isolated peptidoglycan-containing sacculi (lanes d and f). Lanes c, e, and g show supernatants containing the unbound hmwA. All lanes show results from in situ detection of hmwA.

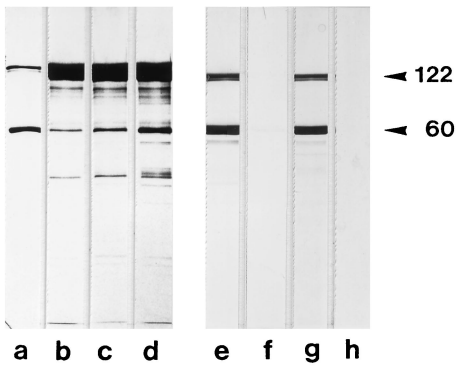

FIG. 6. SDS-PAGE patterns demonstrating the affinity of the S-layer associated protein (P_{60}) to S-layer-carrying cell wall fragments and to isolated peptidoglycan-containing sacculi of *B. stearothermophilus* ATCC 12980. Lane a, supernatant of an S-layer self-assembly suspension containing mainly P_{60}; lane b, S-layer-carrying cell wall fragments; lane c, pellet after incubation of contents of lane a, containing P_{60}, with S-layer-carrying cell wall fragments in Tris-HCl-buffer; lane d, pellet after incubation of contents of lane a, containing P_{60}, with S-layer-carrying cell wall fragments in the presence of 5 M GHCl and dialysis; lane e, self-assembly suspension containing S-layer protein and P_{60}; lane f, isolated peptidoglycan-containing sacculi; lane g, pellet after incubation of contents of lane e, containing S-layer protein and P_{60}, with isolated peptidoglycan-containing sacculi; lane h, supernatant after incubation of contents of lane e, containing S-layer protein and P_{60}, with isolated peptidoglycan-containing sacculi. Values to the right are molecular weights (in thousands).

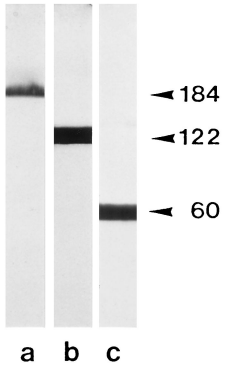

FIG. 7. SDS-PAGE patterns confirming the purity of the hmwA (lane a), the S-layer protein (lane b), and the S-layer-associated protein with a molecular weight of 60,000 (P_{60}) (lane c) used for production of polyclonal rabbit antisera. Values to the right are molecular weights (in thousands).

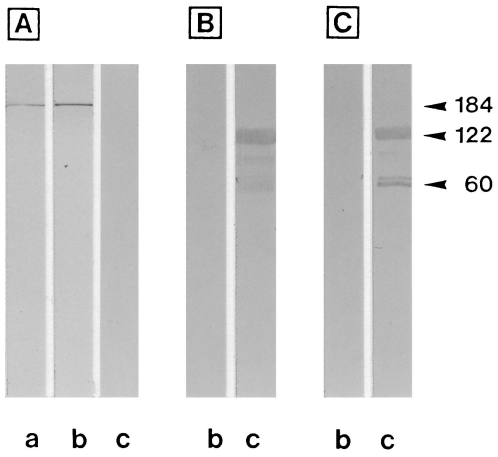

FIG. 8. Immunoreactivities of the hmwA from the S-layer-carrying strain (lane a), the S-layer-deficient variant (lanes b), and the S-layer protein as well as P_{60} (lanes c) from *B. stearothermophilus* ATCC 12980 with polyclonal rabbit antisera against the hmwA (A), the S-layer protein (B), and P_{60} (C) from *B. stearothermophilus* ATCC 12980. For all antisera, a 1:10,000 dilution was used. Values to the right are molecular weights (in thousands).

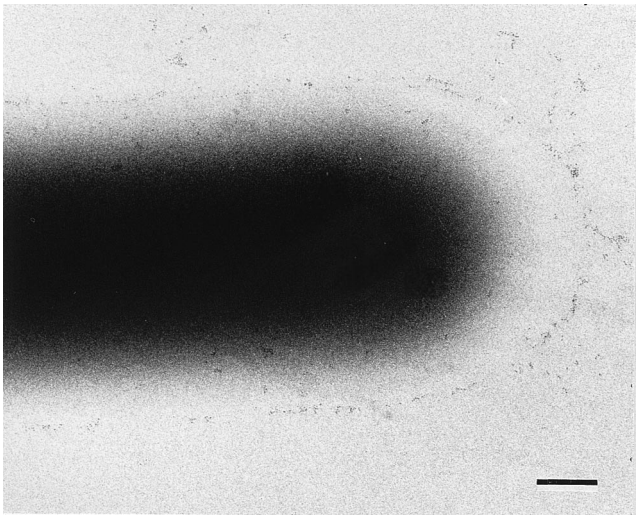

FIG. 10. Protein A-colloidal gold labeling with a polyclonal rabbit antiserum raised against the hmwA on whole cells from the S-layer-carrying strain *B. stearothermophilus* ATCC 12980 grown on complex medium. Bar, 200 nm.

S-layer-deficient variant showed a constant, high level of cell association (Fig. 1A, lanes 3 to 7), and only low concentrations of this enzyme were detected in the culture fluid (data not shown). On the other hand, cell association of the hmwA of the S-layer-carrying strain was strongly dependent on the growth phase of the cells (Fig. 1B, lanes 1 to 7). Two hours after inoculation on complex medium, in the early exponential growth phase, a faint amylase band, which gave a strong reaction when iodine-starch reagent was applied, could be detected on silver-stained SDS gels (Fig. 1B, lane 2). The maximum intensity of cell-associated hmwA, which was comparable to that of the S-layer-deficient variant, was observed 3 h after inoculation (Fig. 1B, lane 3), but it decreased steadily during the subsequent 4 h (Fig. 1B, lanes 4 to 7). In addition to the cell-associated hmwA, a protein band with an apparent molecular weight of 60,000 (P_{60}) appeared on SDS gels 2 h after inoculation (Fig. 1B, lane 2). This protein band showed a greater intensity in whole cells harvested 3 h after inoculation and achieved a maximum 4 to 7 h after inoculation (Fig. 1B, lanes 4 to 7). As long as only small amounts of this protein band could be detected on SDS gels (2 to 3 h after inoculation), the hmwA was found to be associated with the cell pellet (Fig. 1B, lanes 2 and 3). As soon as the intensity of this protein band increased and reached the maximum (4 to 7 hours after inoculation), cell association of the hmwA steadily decreased (Fig. 1B, lanes 4 to 7), and consequently most of the amylase was released into the culture fluid. On the other hand, the hmwA of cells from the S-layer-deficient variant revealed a constant, high level of cell association and the P_{60} could never be observed in SDS extracts of whole cells (Fig. 1A, lanes 3 to 7). Since in SDS extracts from whole cells of the S-layer-carrying organism grown on SVIII medium neither P_{60} nor the hmwA could be detected, there was a strong indication that a relationship between the appearance of P_{60}, the loss of amylase binding sites on whole cells, and the release of the hmwA into the culture fluid exists.

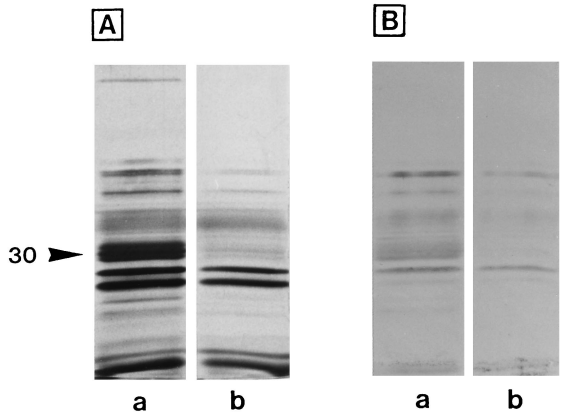

FIG. 9. SDS-PAGE patterns from cleavage products (A) and immunoblots of cleavage products with a polyclonal rabbit antiserum against the S-layer protein of *B. stearothermophilus* ATCC 12980 (B) of the S-layer protein (lanes a) and P_{60} (lanes b) in cell pellets obtained after peptide mapping with endoproteinase Glu-C (*S. aureus* V8 protease). The position of cleavage products of the S-layer protein with molecular weights in the range of 30,000 is marked by the arrow.

Freeze-etching of whole cells of S-layer-carrying strain and S-layer-deficient variant from *B. stearothermophilus* ATCC 12980. In freeze-etched preparations, a highly ordered S-layer lattice with oblique symmetry could be observed on whole cells from the S-layer-carrying strain grown on SVIII medium (Fig. 2b), while the crystalline structure was not visible over wide ranges on stationary growth-phase cells cultivated on complex medium (Fig. 2a). In addition to expressing the hmwA, which at this stage of growth was only present in small amounts in SDS extracts of whole cells, cells grown on complex medium revealed high levels of expression of P_{60} (Fig. 2c). On SDS gels, the intensity of P_{60} was comparable to that of the S-layer protein (Fig. 2c). On the other hand, cells that were cultivated on SVIII medium and did not produce P_{60} (Fig. 2d) clearly exhibited the oblique S-layer lattice (Fig. 2b). Freeze-etched preparations of whole cells from the S-layer-deficient variant revealed an amorphous cell surface, typical of strains lacking an S-layer (data not shown).

Treatment of whole cells from S-layer-carrying and S-layer-deficient variants with different GHCl concentrations for extraction of hmwA. As shown by SDS-PAGE and in situ detection of amylase activity, the hmwA remained associated with

cells from the S-layer-deficient variant even after treatment of whole cells with 2 M GHCl (Fig. 3, lanes c and d). In contrast, treatment of S-layer-carrying cells with 2 M GHCl led to the total extraction of the hmwA without disintegration of the S-layer lattice (data not shown). A complete solubilization of the cell-associated hmwA from both the S-layer-carrying and the S-layer-deficient variants could be achieved by treatment of whole cells with 4 M GHCl (data not shown). These results indicate that the bonds between the hmwA and the cell surface of S-layer-deficient cells are stronger than those between the enzyme and the S-layer lattice.

Preparation of cell wall fragments and S-layer self-assembly products from the S-layer-carrying strain. Cell wall fragments from the S-layer-carrying strain *B. stearothermophilus* ATCC 12980 grown on starch medium were investigated by SDS-PAGE and used for the extraction of the S-layer protein. On SDS gels, a faint band representing the hmwA (M_r 184,000) as well as bands representing the S-layer protein (M_r 122,000) and P_{60} could be identified (data not shown). SDS-PAGE patterns from peptidoglycan-containing sacculi further revealed that both P_{60} and the S-layer protein were completely extracted from cell wall fragments with 5 M GHCl. When GHCl was removed by dialysis, the S-layer protein and P_{60} could be detected in the suspension containing S-layer self-assembly products and nonassembled soluble protein. After sedimentation of S-layer self-assembly products by centrifugation, only small amounts of the S-layer protein (<10% from the total S-layer protein content of the suspension) but at least 50% of P_{60} could be detected in the supernatant (Fig. 4, lane a). The residual 50% of P_{60} was incorporated into S-layer self-assembly products (Fig. 4, lane b). By negative staining, such sheet-like self-assembly products did not exhibit a distinct lattice structure (data not shown).

Studies on interactions between hmwA, P_{60}, and different cell wall components. As demonstrated by SDS-PAGE, the isolated hmwA (Fig. 5, lane a) could bind to S-layer self-assembly products (Fig. 5, lane b) and to the peptidoglycan-containing layer of the S-layer-carrying strain (Fig. 5, lane d) and the S-layer-deficient variant (Fig. 5, lane f) to comparable extents, indicating that the enzyme had affinity to both cell envelope components. The reassociation of the hmwA with S-layer self-assembly products confirmed that the enzyme recognized binding sites on the S-layer surface and was not integrated into the crystal lattice.

Upon adding the S-layer self-assembly fraction containing mainly P_{60} (Fig. 6, lane a) to S-layer-carrying cell wall fragments possessing complete outer and inner S-layers (Fig. 6, lane b), only small amounts of P_{60} were bound (Fig. 6, lane c). On the other hand, P_{60} was integrated into the S-layer lattice which was formed by the recrystallization of S-layer subunits on the surfaces of peptidoglycan-containing sacculi (Fig. 6, lane d). As demonstrated by SDS-PAGE, P_{60} as present in the self-assembly suspension (Fig. 6, lane e) also had high-level affinity to isolated peptidoglycan-containing sacculi (Fig. 6, lanes f to h). These results clearly show that in contrast to the hmwA, which recognized the S-layer surface in S-layer self-assembly products (Fig. 5, lane b), P_{60} was integrated into the S-layer lattice (Fig. 6, lane d). Both proteins could bind to the peptidoglycan-containing layer (Fig. 5, lanes d and f and Fig. 6, lane g).

Investigation of a possible enzymatic activity and final identification of P_{60}. No amylase, protease, or lipase activity could be detected in the self-assembly fraction containing about 50% P_{60} and 50% S-layer protein (data not shown).

The purity of the excised protein bands containing hmwA, the S-layer protein, or P_{60} used for immunization was confirmed by SDS-PAGE (Fig. 7). The immunoblot procedure was carried out to investigate whether the S-layer protein, P_{60}, and hmwA possess structurally homologous domains (Fig. 8). The polyclonal antiserum raised against P_{60} showed a strong reaction with both the S-layer protein and P_{60} (Fig. 8C). On the other hand, the polyclonal antiserum directed against the pure S-layer protein gave a strong immunological signal in response to the S-layer protein but only a weaker cross-reaction with P_{60} (Fig. 8B). Antibodies raised against the hmwA revealed a specific labeling of the enzyme derived from the wild-type strain and the variant but exhibited no cross-reaction with the S-layer protein or P_{60} (Fig. 8A). On immunoblots, the application of preimmune sera showed no nonspecific labeling with any of the proteins used for immunization. Results obtained by immunoblotting strongly indicated that P_{60} and the S-layer protein are structurally related, but no domains homologous between the S-layer protein and the hmwA were detected (Fig. 8). For further comparison, the S-layer protein and P_{60} purified by SDS-PAGE were used for peptide mapping with endoproteinase Glu-C (*S. aureus* V8 protease), which specifically attacks proteins after Asp and Glu residues. As shown in Fig. 9A, both proteins yielded very similar but not identical SDS-PAGE patterns of cleavage products. Interestingly, three cleavage products of the S-layer protein, with molecular weights of 29,000, 30,000, and 31,000, showed higher-level intensities than fragments of the same size from P_{60}.

When SDS-PAGE patterns of cleavage products were investigated by immunoblotting, the three fragments with molecular weights of 29,000, 30,000, and 31,000 from the S-layer protein revealed strong immunological signals with antibodies directed against the S-layer protein but not with antibodies raised against P_{60} (Fig. 9B). Irrespective of differences concerning the immunolabeling of peptides with molecular weights of about 30,000, both antisera showed cross-reactivity with all other cleavage products from the S-layer protein and P_{60} (Fig. 9B).

N-terminal sequencing of the S-layer protein and P_{60} revealed identical N-terminal regions characteristic of S-layer proteins of *B. stearothermophilus* wild-type strains: Ala-Thr-Asp-Val-Ala-Thr-Val-Val-Ser-Gln-Ala-Lys-Ala-Gln. These results from peptide mapping, immunoblotting, and N-terminal sequencing confirmed that P_{60} represents an N-terminal S-layer protein fragment.

Investigation of P_{60} secretion into culture fluid. On SDS gels, a protein with a molecular weight of 60,000 (P_{60}) not only was detected in the cell pellet of the S-layer-carrying strain but was also found in the culture fluid. Since the molecular weight of the N-terminal S-layer protein fragment P_{60} revealed by SDS-PAGE was exactly half that determined for the S-layer protein, the protein detected in the culture fluid could theoretically represent the C-terminal moiety of the S-layer protein obtained by proteolytic cleavage of the S-layer subunits. However, peptide mapping, immunoblotting, and N-terminal sequencing confirmed that P_{60} and the protein with a molecular weight of 60,000 detected in the culture fluid were identical (data not shown).

Immunogold labeling of whole cells of *B. stearothermophilus* ATCC 12980 and the S-layer-deficient variant 12980-9/1. Whole cells from both the S-layer-carrying and the S-layer-deficient variants cultivated on complex medium were used for immunogold labeling, applying the antiserum against hmwA. Although the S-layer-carrying strain as well as the S-layer-deficient variant revealed only weak overall labeling with the antiserum against hmwA, the surface location of the enzyme could clearly be demonstrated (Fig. 10). Electron micrographs of immunogold-labeled cells revealed that cells had collapsed during drying on the grid. The dark central region seen on the

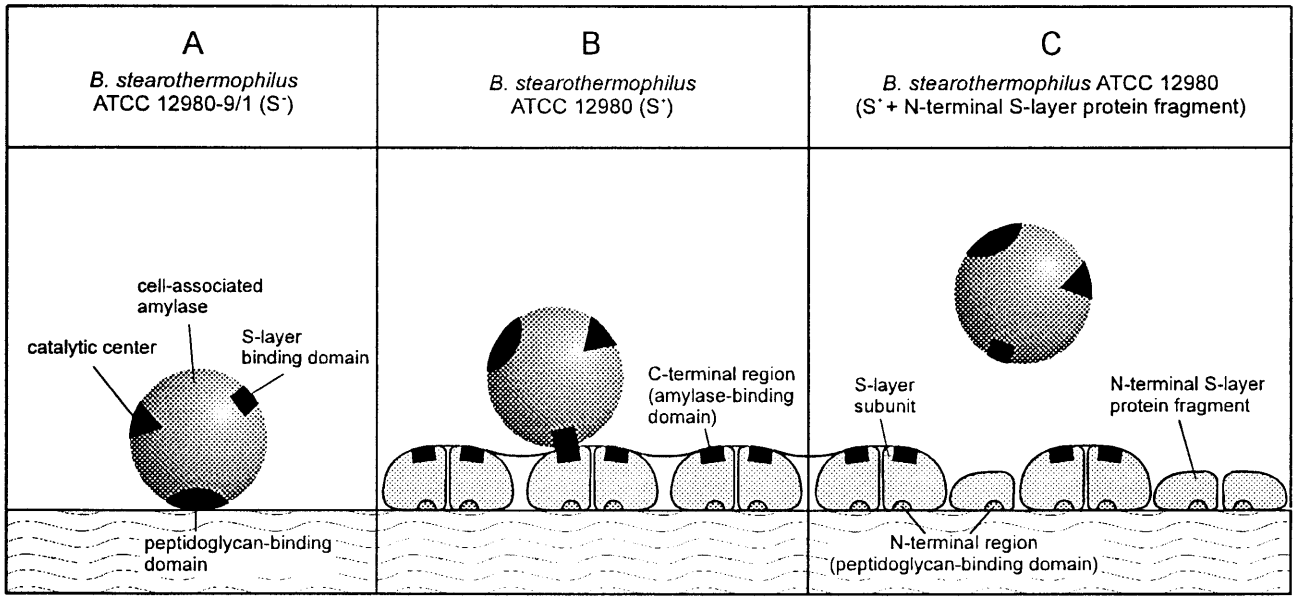

FIG. 11. Hypothetical model as to how the N-terminal S-layer protein fragment triggers the release of the hmwA. (A) In the S-layer-deficient variant (S^-), the hmwA is bound to the cell surface via the peptidoglycan binding domain. (B) In the S-layer-carrying strain (S^+) when the level of expression of the N-terminal S-layer protein fragment (P_{60}) is low, the amylase is bound to the cell surface via the S-layer binding domain located at the C-terminal moiety of the S-layer protein. (C) High-level expression of P_{60}, which leads to the loss of the regular structure of the S-layer lattice, inhibits binding of the hmwA to amylase binding sites on the surface-located C-terminal regions of the S-layer subunits.

micrographs represents the shrunken protoplast which retracted from the cell wall.

Growth of *B. stearothermophilus* ATCC 12980 in the presence of protease inhibitors on complex medium. To rule out the possibility that the N-terminal S-layer protein fragment is a cleavage product of the S-layer protein produced by the cell's own proteases, cells were grown in the presence of different protease inhibitors. As shown by SDS-PAGE of cell pellets and culture supernatants, the addition of different inhibitors for serine proteases, cysteine proteases, metalloproteases, metalloendopeptidases, amino peptidases, and aspartate proteases had no influence on the presence of P_{60} in the cell pellet and in the culture fluid (data not shown).

DISCUSSION

In this study the role of the S-layer lattice with regard to exoprotein secretion was investigated by using the S-layer-carrying strain *B. stearothermophilus* ATCC 12980 and the S-layer-deficient variant 12980-9/1 as a model system. It has frequently been observed that S-layers are lost upon prolonged cultivation under optimal laboratory conditions, thus supporting the assumption that they endow the living cell with an advantage of selection in competitive natural habitats (22). Several data indicate that lack of S-layer gene expression in spontaneous mutants is caused by deletion of promoter sequences (15). For the closely related *B. stearothermophilus* PV72, hybridization and PCR analysis suggested that in the S-layer-deficient variant T5, the 5′ region of the S-layer gene *sbsA* has a deletion of approximately 800 bp compared with the wild-type strain (26).

Both the S-layer-carrying strain *B. stearothermophilus* ATCC 12980 and the S-layer-deficient variant 12980-9/1 were shown to secrete three amylases, with molecular weights of 58,000, 122,000, and 184,000, into the culture fluid. As previously demonstrated for *B. stearothermophilus* DSM 2385, only the hmwA was found to be cell associated (7). The S-layer-carrying strain and the variant lacking the S-layer revealed differences regarding the secretion and cell association of the hmwA. In contrast to the S-layer-carrying strain, the S-layer-deficient variant showed constant levels of cell-associated and free amylase. In the S-layer-carrying strain, the amount of cell-associated amylase strongly depended on the growth phase of the cells. In addition to the S-layer protein, another prominent protein band with a molecular weight of 60,000 (P_{60}) appeared on SDS gels when the cells were grown on starch medium. High-level expression of P_{60} by S-layer-carrying cells grown on starch medium was accompanied by a reduced cell association and release of the hmwA into the culture fluid.

The results from N-terminal sequencing, peptide mapping, and immunoblotting revealed that P_{60} is a truncated S-layer protein devoid of the C-terminal region as already described for a Tn*5* insertion mutant of *Aeromonas hydrophila* TF7 and for *Campylobacter fetus* (35, 36). Since the possibility that P_{60} is a proteolytic cleavage product of the whole S-layer protein can be excluded from our experimental data, we suggest that this N-terminal S-layer protein fragment is the product of a truncated copy of the S-layer gene which is coexpressed with the hmwA gene under certain culture conditions. Induction is obviously triggered together with the hmwA on the starch-containing medium. As shown by affinity studies, P_{60} retained the ability to bind to isolated peptidoglycan-containing sacculi.

B. stearothermophilus wild-type strains were shown to possess identical peptidoglycan types, comparable amounts and compositions of secondary cell wall polymers, and identical N-terminal regions of their S-layer proteins (26). Extraction and recrystallization experiments strongly indicated that the S-layer proteins recognize the secondary cell wall polymers as specific binding sites. For *B. stearothermophilus* ATCC 12980 it could be demonstrated that reassociation of the N-terminal S-layer protein fragment P_{60} to S-layer-carrying cell wall fragments was possible only after disintegration of the S-layer lattice with 5 M GHCl and dialysis. This clearly showed that

association of P_{60} with the S-layer is caused not by mere surface adhesion but by integration into the crystal lattice. Since P_{60} could also bind to isolated peptidoglycan-containing sacculi, it was confirmed that the N-terminal part of the S-layer protein anchors the subunits to the rigid cell wall layer.

Extraction experiments with different GHCl concentrations revealed that in S-layer-carrying *B. stearothermophilus* ATCC 12980 cells, total removal of the hmwA with 2 M GHCl without disintegration of the S-layer lattice was possible. On the other hand, the hmwA could not be liberated from the peptidoglycan-containing layer in S-layer-deficient cells under these conditions.

Since the hmwA had affinity to both the S-layer surface and the peptidoglycan-containing layer, it can be assumed that this amylase carries two different binding domains, one for association with the S-layer surface and one for binding to the peptidoglycan-containing layer. The hmwA from *B. stearothermophilus* ATCC 12980 did not cross-react with the S-layer protein on immunoblots, indicating that the enzyme and the S-layer protein do not possess structurally homologous domains. These results are in accordance with those obtained for the hmwA from *B. stearothermophilus* DSM 2358 (7).

On the basis of the experimental data provided in our study, we suggest the following model as to how the N-terminal S-layer protein fragment P_{60} triggers the release of the hmwA. As proposed in Fig. 11, the hmwA carries both a peptidoglycan and an S-layer binding domain. In the S-layer-deficient variant, hmwA is bound to the cell surface via the peptidoglycan binding domain. Since P_{60} has no affinity to the hmwA and in complete S-layer subunits represents the domain responsible for the specific binding of the S-layer subunits to the rigid cell wall layer, the C-terminal region of the S-layer protein must function as the binding site for the hmwA. The expression of P_{60}, which leads to the loss of the regular structure of the S-layer lattice because of incorporation into the crystal lattice, can obviously block the amylase binding sites on the surface-located C-terminal region of the S-layer subunits.

ACKNOWLEDGMENTS

This work was supported by the Austrian Science Foundation (project S72/02) and by the Federal Ministry of Science, Research, and the Arts, Republic of Austria.

We thank Christoph Hotzy for excellent technical assistance and Karola Vorauer-Uhl for N-terminal sequencing.

REFERENCES

1. **Archibald, R.** 1989. The *Bacillus* cell envelope. Biotechnol. Handb. **2:**217–254.
2. **Bartelmus, W., and F. Perschak.** 1957. Schnellmethode zur Keimzahlbestimmung in der Zuckerindustrie. Z. Zuckerind. **7:**276–281.
3. **Beveridge, T. J.** 1994. Bacterial S-layers. Curr. Opin. Struct. Biol. **4:**204–212.
4. **Beveridge, T. J., and L. L. Graham.** 1991. Surface layers of bacteria. Microbiol. Rev. **55:**684–705.
5. **Beveridge, T. J., and S. F. Koval (ed.).** 1993. Advances in bacterial paracrystalline surface layers. Plenum Publishing Corp., New York.
6. **Breitwieser, A., K. Gruber, and U. B. Sleytr.** 1992. Evidence for an S-layer pool in the peptidoglycan of *Bacillus stearothermophilus*. J. Bacteriol. **174:** 8008–8015.
7. **Egelseer, E., I. Schocher, M. Sára, and U. B. Sleytr.** 1995. The S-layer from *Bacillus stearothermophilus* DSM 2358 functions as an adhesion site for a high-molecular-weight amylase. J. Bacteriol. **177:**1444–1451.
8. **Engel, A. M., Z. Cejka, A. Lupas, F. Lottspeich, and W. Baumeister.** 1992. Isolation and cloning of Ompα, a coiled-coil protein spanning the periplasmic space of the ancestral eubacterium *Thermotoga maritima*. EMBO J. **11:**4369–4378.
9. **Faraldo, M. L., M. A. de Pedros, and J. Berenguer.** 1988. Purification, composition and Ca^{2+}-binding properties of the monomeric protein of the S-layer of *Thermus thermophilus*. FEBS Lett. **235:**117–121.
10. **Fujino, T., P. Béguin, and J.-P. Aubert.** 1993. Organization of a *Clostridium thermocellum* gene cluster encoding the cellulosomal scaffolding protein CipA and a protein possibly involved in attachment of the cellulosome to the cell surface. J. Bacteriol. **175:**1891–1899.
11. **Gowland, P., M. Kernick, and T. K. Sundaram.** 1987. Thermophilic bacterial isolates producing lipase. FEMS Microbiol. Lett. **48:**339–343.
12. **Graham, L. L., T. J. Beveridge, and N. Nanninga.** 1991. Periplasmic space and the concept of periplasm. Trends Biochem. Sci. **16:**328–329.
13. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Hastie, A. T., and C. C. Brinton, Jr.** 1979. Specific interaction of the tetragonally arrayed protein layer of *Bacillus sphaericus* with its peptidoglycan sacculus. J. Bacteriol. **138:**1010–1021.
15. **Kuen, B., and W. Lubitz.** 1996. Analysis of S-layer proteins and genes, p. 77–102. *In* U. B. Sleytr, P. Messner, D. Pum, and M. Sára (ed.), Crystalline bacterial cell surface proteins. R. G. Landes Co. and Academic Press, Austin, Tex.
16. **Lacks, S. A., and S. S. Springhorn.** 1980. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. J. Biol. Chem. **255:**7467–7473.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Lemaire, M., H. Ohayon, P. Gounon, T. Fujino, and P. Béguin.** 1995. OlpB, a new outer layer protein of *Clostridium thermocellum*, and binding of its S-layer-like domains to components of the cell envelope. J. Bacteriol. **177:** 2451–2459.
19. **Lupas, A., H. Engelhardt, J. Peters, U. Santarius, S. Volker, and W. Baumeister.** 1994. Domain structure of the *Acetogenium kivui* surface layer revealed by electron crystallography and sequence analysis. J. Bacteriol. **176:**1224–1233.
20. **Matuschek, M., G. Burchhardt, K. Sahm, and H. Bahl.** 1994. Pullulanase of *Thermoanaerobacterium 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.
21. **Messner, P., F. Hollaus, and U. B. Sleytr.** 1984. Paracrystalline cell wall surface layers of different *Bacillus stearothermophilus* strains. Int. J. Syst. Bacteriol. **34:**202–210.
22. **Messner, P., and U. B. Sleytr.** 1992. Crystalline bacterial cell surface layers. Adv. Microb. Physiol. **33:**213–275.
23. **Priest, F. G.** 1981. Products and applications. Biotechnol. Handb. **2:**293–320.
24. **Salamitou, S., M. Lemaire, T. Fujino, H. Ohayon, P. Gounon, P. Béguin, and J.-P. Aubert.** 1994. 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.
25. **Salamitou, S., O. Raynaud, M. Lemaire, M. Coughlan, P. Béguin, and J.-P. Aubert.** 1994. Recognition specificity of the duplicated segments present in *Clostridium thermocellum* endoglucanase CelD and in cellulosome-integrating protein CipA. J. Bacteriol. **176:**2822–2827.
26. **Sára, M., B. Kuen, H. F. Mayer, F. Mandl, K. C. Schuster, and U. B. Sleytr.** 1996. Dynamics in oxygen-induced changes in S-layer protein synthesis and cell wall composition in continuous culture from *Bacillus stearothermophilus* PV72 and the S-layer-deficient variant T5. J. Bacteriol. **178:**2108–2117.
27. **Sára, M., and U. B. Sleytr.** 1987. Production and characteristics of ultrafiltration membranes with uniform pores from two dimensional arrays of proteins. J. Membr. Sci. **33:**27–49.
28. **Sleytr, U. B., and A. M. Glauert.** 1975. Self-assembly of the hexagonally and tetragonally arranged subunits of bacterial surface layers and their reattachment to cell walls. J. Ultrastruct. Res. **55:**360–377.
29. **Sleytr, U. B., P. Messner, D. Pum, and M. Sára (ed.).** 1988. Crystalline bacterial cell surface layers. Springer-Verlag, Berlin.
30. **Sleytr, U. B., P. Messner, D. Pum, and M. Sára.** 1993. Crystalline bacterial cell surface layers. Mol. Microbiol. **10:**911–916.
31. **Sleytr, U. B., P. Messner, D. Pum, and M. Sára (ed.).** 1996. Crystalline bacterial cell surface proteins. R. G. Landes Co. and Academic Press, Austin, Tex.
32. **Specka, U., A. Spreinat, G. Antranikian, and F. Mayer.** 1991. Immunocytochemical identification and localization of active and inactive α-amylase and pullulanase in cells of *Clostridium thermosulfurogenes* EM1. Appl. Environ. Microbiol. **57:**1062–1069.
33. **Sturm, E., E. Egelseer, M. Sára, and U. B. Sleytr.** 1993. Can S-layers of *Bacillaceae* control the release of their own exoproteins?, p. 297–302. *In* T. J. Beveridge and S. F. Koval (ed.), Advances in bacterial paracrystalline surface layers. Plenum Publishing Corp., New York.
34. **Takekawa, S., N. Uozumi, N. Tsukagoshi, and S. Udaka.** 1991. Proteases involved in generation of β- and α-amylases from a large amylase precursor in *Bacillus polymyxa*. J. Bacteriol. **173:**6820–6825.
35. **Trust, T. J.** 1993. Molecular, structural and functional properties of *Aeromonas* S-layers, p. 159–171. *In* T. J. Beveridge and S. F. Koval (ed.), Advances in bacterial paracrystalline surface layers. Plenum Publishing Corp., New York.
36. **Yang, L., Z. Pei, S. Fujimoto, and M. J. Blaser.** 1992. Reattachment of surface array proteins to *Campylobacter fetus* cells. J. Bacteriol. **174:**1258–1267.