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Horizontal Transference of S-Layer Genes within *Thermus thermophilus*

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The S-layers of *Thermus thermophilus* HB27 and *T. thermophilus* HB8 are composed of protein units of 95 kDa (P95) and 100 kDa (P100), respectively. We have selected S-layer deletion mutants from both strains by complete replacement of the *slpA* gene. Mutants of the two strains showed similar defects in growth and morphology and overproduced an external cell envelope inside of which cells remained after division. However, the nature of this external layer is strain specific, being easily stained and regular in the HB8Δ*slpA* derivative and amorphous and poorly stained in the HB27Δ*slpA* strain. The addition of chromosomic DNA from *T. thermophilus* HB8 to growing cultures of *T. thermophilus* HB27Δ*slpA* led to the selection of a new strain, HB27C8, which expressed a functional S-layer composed of the P100 protein. Conversely, the addition of chromosomic DNA from *T. thermophilus* HB27 to growing cultures of *T. thermophilus* HB8Δ*slpA* allowed the isolation of strain HB8C27, which expressed a functional S-layer composed of the P95 protein. The driving force which selected the transference of the S-layer genes in these experiments was the difference in growth rates, one of the main factors leading to selection in natural environments.

In many mesophilic bacteria, the presence of crystalline surface layers (S-layers) seems to be a strain-specific character, whose loss upon optimal growth conditions results in S-layer mutants that do not present any apparent phenotypic defect (12, 21). By contrast, most thermophiles, especially those belonging to the oldest phylogenetic branches (31), contain S-layer as an almost universal character, thus suggesting for these structures important roles in cell viability or membrane integrity at high temperatures. In fact, the presence of S-layer in evolutionarily old thermophilic bacteria and their structural simplicity led to the suggestion of an ancient evolutionary origin for such structures (26).

As S-layers completely surround the cells, their building units constitute one of the major membrane proteins. Accordingly, synthesis of the S-layer is a metabolically expensive process which could be selected during evolution only as a result of the existence of strong selective pressures. However, little is known about such selective pressures, and even less is known about the role(s) that S-layers could play in mesophilic or thermophilic natural environments.

Like any cell surface components, S-layers should be subjected to strong selective pressures, the most important of which could be the presence of hydrolytic enzymes (essentially proteases) and bacteriophages (as binding sites). Such selective pressures could be the factors responsible for the sequence divergence of the S-layer genes (21). In fact, it has been not possible to obtain clear phylogenetic relationships between S-layer genes, even from related organisms. In addition, the possibility exists that horizontal transference of S-layer genes within genetically related strains contributed to the present sequence divergence, in a way similar to that described for other bacterial membrane protein genes also subjected to strong selective pressures (28, 30).

To determine if the horizontal transference of S-layer genes could occur in extreme thermophiles, we have selected *Thermus thermophilus* HB8 (23) and *T. thermophilus* HB27 (13) as models. These strains were chosen because they have two essential properties. First, like other isolates belonging to genus *Thermus*, these strains are able to incorporate linear DNA from the culture medium into their chromosomes (13). The second property, essential for these assays, is the presence of typical S-layers in both strains (reference 3 and this work) whose building units can be easily distinguished through differences in electrophoretical mobility.

The hexagonal S-layer of *T. thermophilus* HB8 (3–5) is composed of a 100-kDa protein (P100) which has been previously characterized (6). Its coding gene, *slpA* (for S-layer protein) has been cloned and sequenced (7, 8), and its promoter has been found to function in both *Thermus* spp. and *Escherichia coli* (8). By using this promoter and a thermostable kanamycin resistance gene (20), we developed a selectable gene marker for *T. thermophilus* (at 70°C) and *E. coli* (at 37°C) (15). In previous work, the use of this marker allowed us to demonstrate the important role that the S-layer plays in *T. thermophilus* HB8. These results were the basis of the work presented in this article.

T. thermophilus HB27 is essentially used as a host for the development of genetic manipulation systems in extreme thermophiles (13, 16, 19). As we describe in this article, this strain also contains an S-layer, revealed both by the presence of a 95-kDa major cell envelope protein (P95) and by direct microscopic detection of hexagonal crystals in cell envelope fractions treated with detergents and EDTA as described previously (4). The difference in molecular size between the S-layer proteins from *T. thermophilus* HB8 (100 kDa) and *T. thermophilus* HB27 (95 kDa) should allow us to detect any horizontal transference of the respective coding genes.

In this article, we describe the isolation of mutants from *T.*

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thermophilus HB27 and HB8 with complete deletions in the corresponding S-layer genes, and we demonstrate that *slpA* genes can be transferred and expressed as functional elements on cell envelopes in both strains. As we show here, the selection pressure which drives such transference is a difference in growth rate, one of the primary forces in natural environments.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages and growth conditions. *T. thermophilus* HB8 (ATCC 27634) was obtained from the American Type Culture Collection (Rockville, Md.), and *T. thermophilus* HB27 was generously provided by Y. Koyama. *E. coli* DH5αF′ [F′ *supE44* Δ(*lacZYA-arF*)U169 (F80 *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 laqI*^{q}*Z*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] (32) were used as hosts for genetic manipulations of plasmids.

T. thermophilus HB8 was 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. 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 plates for selection. Plates were incubated at 70°C in a water-saturated atmosphere.

E. coli strains were grown at 37°C with aeration in M9 minimal medium (22), supplemented with the specific requirements, or in LB medium (17). If 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 previously (10).

pUC119 plasmids (29) were used for in vitro genetic constructions. pUC119ΔS (this work) is a derivative of pUC119 in which the *Sal*I restriction site has been deleted. Plasmids pMF4 (7), pKT1 (15), and pRCS1.KB (9) were previously described. Phage λRCS1.0 was obtained from a *T. thermophilus* HB8 library as described previously (9).

Transformation of *T. thermophilus*. The method used for plasmid transformation was essentially that of Koyama et al. (13). *T. thermophilus* strains were grown at 70°C in a transformation medium, which contained, per liter of tap water, 4 g of Trypticase (BBL), 2 g of yeast extract (Oxoid), 1.5 g of NaCl, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$ (pH 7.5). At an optical density at 550 nm of 0.3, samples of 0.5 ml were transferred to 5-ml sterile tubes, and the desired amount of DNA was added. After 2 h of incubation at 70°C under strong aeration, cells were directly plated onto selective agar plates.

For assays of horizontal transference of *slpA* genes, 10 μg of chromosomic DNA was directly added to 20 ml of exponential cultures (optical density at 550 nm = 0.3) growing at 70°C in the same medium. Cultures were maintained in exponential growth through 1/100 dilution in preheated rich medium.

DNA analysis. Most DNA techniques used in this study were carried out as described by Sambrook et al. (24). Plasmid DNA was purified from *E. coli* by the alkaline lysis method (2). Total DNA from *T. thermophilus* strains was purified by the method of Marmur (18). Restriction enzymes were used and ligation reactions were performed as described by the manufacturer (Boehringer-Mannheim GmbH, Penzberg, Germany). DNA sequencing was performed by the method of Sanger et al. (25), using 7-deaza-dGTP, modified T7 DNA polymerase (Sequenase 2.0; U.S. Biochemical), [α-^{35}S]dATP (1,000 Ci/mmol; Amersham), and specific oligonucleotides synthesized by Isogen Bioscience (Maarseen, Holland). Uniformly ^{32}P-labeled DNA probes were generated from appropriate DNA templates 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.

Southern blot analysis. Southern blot analysis was performed essentially as described by Sambrook et al. (24). Purified DNA (5 μg) was subjected to digestion with restriction enzymes and electrophoresed on agarose gels (0.8% [wt/vol]). DNA was capillary transferred for 16 h to a nylon membrane (Hybond N; Amersham) in 20× SSC (1× SSC is 0.1 M NaCl plus 0.015 M sodium citrate) buffer. Hybridization was carried out for 16 h at 42°C, using ~4 × 10^6 dpm of appropriate ^{32}P-labeled DNA probe produced by random priming (see above) in 10 ml of hybridization solution (6× SSC–0.1% sodium dodecyl sulfate [SDS]–100 μg of denatured salmon sperm DNA per ml). The membrane was washed in 2× SSC–0.1% SDS at 62°C and autoradiographed at −70°C. The probes used were a 0.76-kbp *Nde*I-*Bgl*II fragment purified from plasmid pRCS1.KB (9) for *glmS*, a 0.8-kbp *Nde*I-*Sma*I fragment purified from plasmid pKT1 (15) for *kat* (kanamycin adenyltransferase, Kan^r), and a 0.78-kbp *Nde*I-*Pst*I fragment from plasmid pRCS1.BBΔS (this study) for *slpA*.

Protein analysis and Western blotting (immunoblotting). Cell envelopes were purified as described for *E. coli* (27) and routinely stored at −20°C in 10 mM Tris-HCl (pH 7.8) buffer at a protein concentration of about 30 mg/ml. Cell envelope batches were thawed at room temperature immediately before use. Total proteins from these fractions were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) (10% polyacrylamide gel) as described previously (1).

A collection of monoclonal antibodies was obtained by using P100 protein extracted from SDS-polyacrylamide gels as the antigen (unpublished data). Six of them, which recognize different regions of the S-layer protein, were pooled and used for Western blot experiments. Immunodetection of proteins was performed with an enhanced chemiluminescence Western blotting analysis system (Amersham International).

Electron microscopy. Thin sections of whole cells were obtained after fixation with 2% glutaraldehyde and 2% tannic acid in phosphate-buffered saline (PBS) for 1 h at room temperature. Once extensively washed with PBS, the samples were postfixed with 1% OsO_4 in PBS for 1 h at 4°C, dehydrated in ethanol, and embedded in Epon 812 (Fluka Chemie AG) as described by Lasa et al. (15). Thin sections were stained with lead citrate and washed with 20 mM NaOH and distilled water.

S-layer crystals were obtained from whole cell envelope fractions by treatment with EDTA and Triton X-100 and negatively stained as described previously (4). Electron micrographs were obtained with a JEOL 100B or JEOL 1200EX electron microscope.

RESULTS

Isolation of S-layer deletion mutants of *T. thermophilus* HB8. To delete most of the *slpA* coding sequence from *T. thermophilus* HB8, we developed the strategy depicted in Fig. 1. A 4.8-kbp *Bam*HI-*Bam*HI fragment containing *slpA* was isolated from lambda phage λRCS1.0 (9) and cloned in pUC119ΔS. From the plasmid obtained, pRCS1.BBΔS, most of the coding region of *slpA* (amino acids 1 to 730) was deleted by digestion with restriction enzymes *Acc*I and *Nde*I and replaced by the thermostable *kat* gene contained in a 0.7-kbp *Nde*I-*Sma*I fragment from pKT1 (15). The new plasmid, pKBB, contained a 1.5-kbp region downstream of the *slpA* transcription terminator and a functional kanamycin resistance gene expressed from the bifunctional *E. coli-Thermus slpA* promoter. To provide also regions upstream of *slpA*, the 3.4-kbp *Bam*HI-*Bam*HI fragment from plasmid pKBB, which contains the deleted form of *slpA*, was cloned into the *Bam*HI site of plasmid pRCS1.KB (9), creating plasmid pKKB. This plasmid was used for in vivo gene replacement experiments.

Plasmid pKKB (10 μg) was then used to transform *T. thermophilus* HB8 as described in Materials and Methods. After 2 days of incubation at 70°C, about 30 colonies grew on plates containing 30 μg of kanamycin per ml; 24 of these colonies were grown at 70°C in 1 ml of rich medium and analyzed by optical microscopy. Of the 24 clones analyzed, 7 showed aberrant phenotypes such as aggregation, curly morphology, and the presence of common cell envelopes surrounding groups of cells. The absence of P100 in these colonies was further confirmed by SDS-PAGE (data not shown).

To confirm that the construction obtained in vitro was properly inserted into the chromosomes of these mutants, total DNA from four of them was purified, digested with restriction enzymes *Bam*HI and *Kpn*I, and subjected to Southern blot analysis. The results of hybridization with probes for genes *slpA*, *kat*, and *glmS* (glucosamine synthase) are shown in Fig. 2. As can be seen, the use of the *slpA* probe allowed the detection of the expected 6.3-kbp *Kpn*I and 4.7-kbp *Bam*HI fragments in the wild-type strain but not in any of the mutants. Conversely, the use of the *kat* probe allowed the detection of a 5.1-kbp *Kpn*I and a 3.5-kbp *Bam*HI fragment in the mutants but not in the wild type. Finally, the *glmS* probe allowed the identification of a 4.1-kbp *Bam*HI fragment in both wild-type and mutant strains (a result which demonstrated that the regions upstream of *slpA* were preserved) and a *Kpn*I fragment (5.1 kbp) smaller than that of the wild type (6.3 kbp). These results clearly demonstrated that all of the mutants checked were obtained through the expected double-recombination event and thus carried the *slpA* deletion (see the map in Fig. 2). In addition, the results also demonstrated that the phenotypic defects exhibited by the mutants were due to the *slpA* deletion and not to the presence of S-layer fragments or to any polar effects of the *kat* gene on other genes. These mutants were named HB8Δ*slpA*-1 to HB8Δ*slpA*-4.

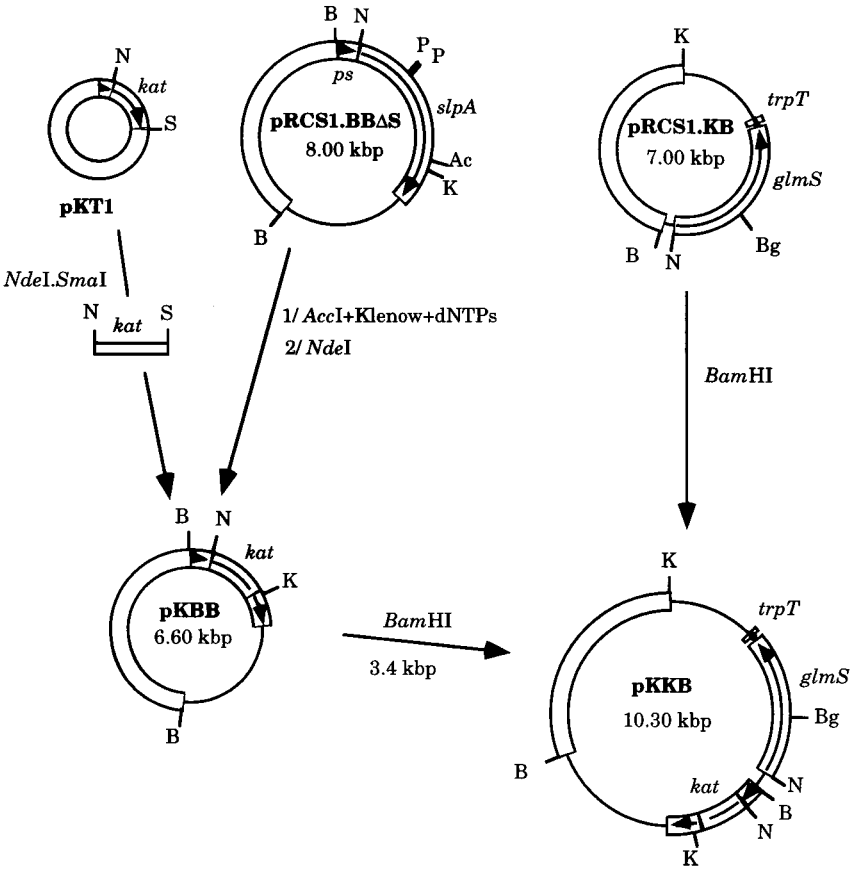

FIG. 1. In vitro replacement of the *T. thermophilus* HB8 S-layer gene. The construction of plasmid pKKB is diagramed. Positions and directions of transcription (arrows) of genes *slpA*, *glmS*, *kat*, and *trpT* (tryptophan tRNA) are labeled. Restriction enzyme abbreviation: Ac, *Acc*I; B, *Bam*HI; Bg, *Bgl*II; K, *Kpn*I; N, *Nde*I; P, *Pst*I. Only sites relevant to this study are indicated. dNTPs, deoxynucleoside triphosphates.

Isolation of S-layer deletion mutants from *T. thermophilus* HB27. Chromosomal DNA (5 μg) from *T. thermophilus* HB8Δ*slpA*3 was used to transform *T. thermophilus* HB27 as described in Materials and Methods, and kanamycin-resistant colonies were selected as described above. Microscopic analysis of these colonies allowed us to detect in most of them phenotypes similar to those described for HB8Δ*slpA* mutants. These mutants were isolated again on plates containing kanamycin (30 μg/ml) and designated HB27Δ*slpA*.

Horizontal transference of S-layer genes. As an initial step in their characterization, S-layer deletion mutants from both strains were shown to grow at around half the rate of their corresponding parental wild-type strains (Fig. 3). This low growth rate allowed us to design an experiment in which chromosomal DNA samples (10 μg) from wild-type *T. thermophilus* (HB8 or HB27) were added to exponential cultures of HB8Δ*slpA*-3 or HB27Δ*slpA*-1 growing in 20 ml of transformation medium (Materials and Methods). Cultures were then maintained in exponential growth phase through serial 1:100 dilutions in preheated (70°C) rich medium. Dilutions were repeated (four times) until microscopic analysis showed that most cells had a normal morphology. Cultures were then plated in the absence of kanamycin, and the colonies obtained were subsequently checked for the loss of kanamycin resistance. About 95 to 98% of the colonies were kanamycin sensitive, suggesting that in each experiment, a strain in which the resistance marker was replaced through a double-recombination process was selected. One isolate from each of these processes was selected for use in further studies; the isolates thus obtained were named HB8C8 (HB8Δ*slpA* transformed with DNA from HB8), HB8C27 (HB8Δ*slpA* transformed with DNA from HB27), and HB27C8 (HB27Δ*slpA* transformed with DNA from HB8).

Colonies from each of these strains were inoculated in rich medium, and their growth was compared with that of their parental wild-type strains and Δ*slpA* mutants (HB8Δ*slpA*-3 and HB27Δ*slpA*-1). The results of this experiment (Fig. 3) demonstrated that the strains selected (HB8C8, HB8C27, and HB27C8) had growth rates indistinguishable from those of their parental wild-type strains. These results also showed that the growth rates of the strains did not depend on the origin of the chromosomal DNA used for genetic transference.

Protein analysis of the strains. Whole envelope fractions from these strains (wild type, S-layer mutants, and complemented derivatives) were purified, and their protein patterns were analyzed. Coomassie blue staining of the gels (Fig. 4A) demonstrated the presence in HB8C8, HB8C27, and HB27C8 of major proteins, whose electrophoretic mobilities were identical to that of the S-layer monomer from the DNA donor strain: a 100-kDa protein (P100; lane 3) in HB8C8, a 95-kDa protein (P95; lane 4) in HB8C27, and a 100-kDa protein (P100; lane 7) in HB27C8. These results strongly suggested that homologous (lane 3) or heterologous (lanes 4 and 7) S-layer proteins could be expressed in both strains.

To confirm that the major proteins expressed in HB8C8, HB8C27, and HB27C8 were identical to the S-layer proteins

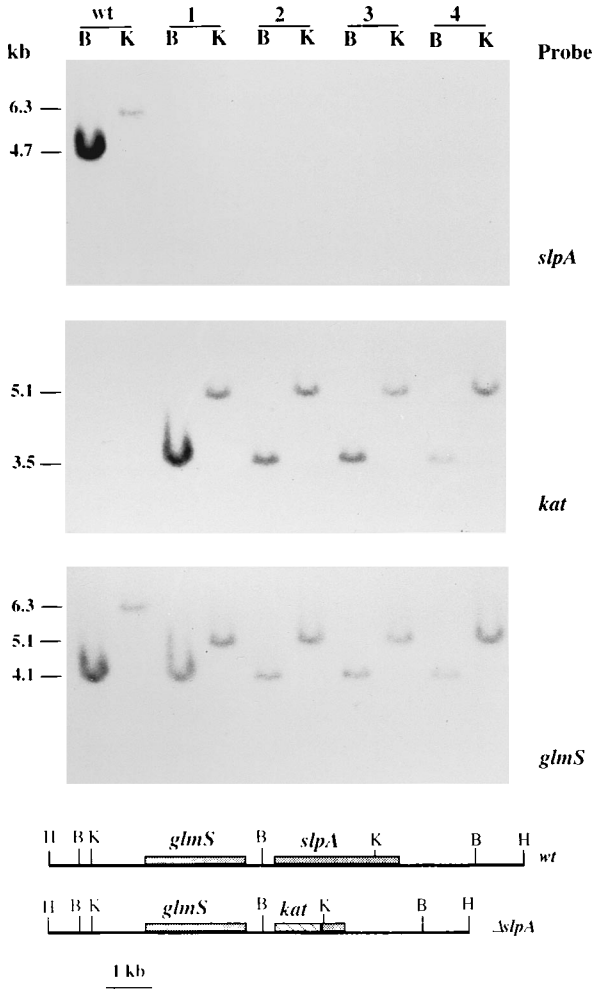

FIG. 2. Southern blot analysis of *T. thermophilus* HB8Δ*slpA*. Total DNA from *T. thermophilus* HB8Δ*slpA* mutants was purified, digested with restriction enzyme *Bam*HI (lanes B) or *Kpn*I (lanes K), treated for Southern blot analysis, and sequentially labeled with probes corresponding to coding regions of genes *slpA*, *kat*, and *glmS* (see the text for details). The sizes and positions of the labeled fragments are indicated. The *Kpn*I (K) and *Bam*HI (B) cutting sites on the chromosome of the wild type (wt) and deletion mutants are shown at the bottom.

from the DNA donor strains, we performed Western blotting with a mixture of six monoclonal antibodies directed against different regions of the *T. thermophilus* HB8 S-layer protein. As shown in Fig. 4B, this antibody mixture recognized S-layer proteins from both wild-type strains, HB8 (P100) and HB27 (P95) (lanes 3 and 4). Furthermore, both P95 (HB27) and P100 (HB8) have size-specific proteolytic fragments even when expressed in different strains. Thus, the presence of specific proteolytic fragments allowed clear identification of the protein expressed: the major protein expressed in HB8C27 (lane 4) corresponds to the 95-kDa S-layer protein from HB27 (lane 5), and that expressed by HB27C8 (lane 7) corresponds to the 100-kDa S-layer protein from HB8 (lane 1).

In the experiment shown in Fig. 4A, it was observed that concomitantly with the deletion of the *slpA* protein, three major low-molecular-mass proteins (52, 50, and 36 kDa) were induced in HB8Δ*slpA* (lane 2). An identical protein pattern was previously observed in *slpA* insertional mutants (15). However, in those experiments, we suggested that the new major proteins were expressed from fragments of *slpA*. Nevertheless, the nature of the construction used to obtain the HB8Δ*slpA* strain and the inability to detect these bands with the monoclonal antibodies against P100 (Fig. 4B, lane 6) clearly demonstrated that these proteins were synthesized from a different locus. In addition, the induction of these proteins seemed to be a strain-specific characteristic, as similar proteins were not detected in any of the HB27Δ*slpA* mutants checked (Fig. 4A, lane 6).

Electron microscopy of *slpA* mutants and complemented strains. The experiment described above clearly demonstrated that different S-layer genes could be transferred and expressed in *T. thermophilus* HB8 and HB27. To determine whether these proteins were functional S-layers when expressed in the heterologous strain, we analyzed by electron microscopy thin sections of wild-type, mutant, and complemented strains.

Deletion of the S-layer in HB8Δ*slpA* and HB27Δ*slpA* led to loss of the external layer and the thick underlying material (Fig. 5b and e). Concomitantly, groups of cells appeared surrounded by common layers whose composition, although unknown, was clearly different in each strain. The common envelope was well stained and regular in HB8Δ*slpA* (Fig. 5b, arrowhead). By contrast, the common envelope in HB27Δ*slpA* stained poorly and did not have any regular pattern (Fig. 5e).

Thin sections of strains HB8C27 and HB27C8 are almost indistinguishable from those of the corresponding parental wild-type strains. However, the presence of a regular external layer could be detected only when the P100 protein was expressed (Fig. 5a and f), while we could not detect any regular structure (from HB27) on the cell surface with expression of the P95 protein (Fig. 5d and c). These results suggest that the P95 protein was unable to form an S-layer even on strain HB27. To check this possibility, we purified total cell envelopes from strain HB8C27 and treated them as described previously

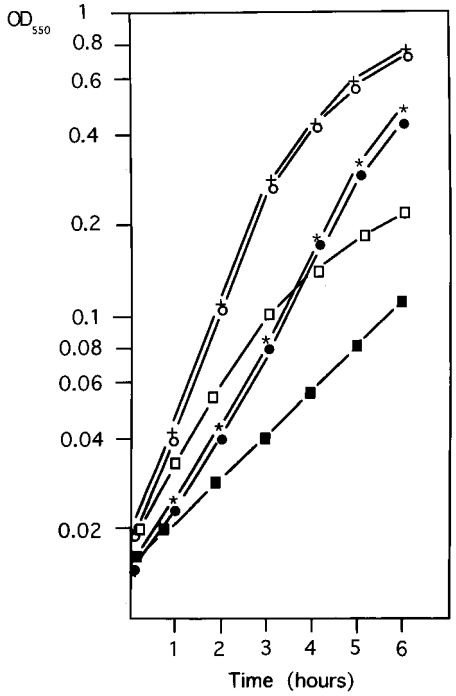

FIG. 3. Growth of *T. thermophilus* strains. The optical densities at 550 nm (OD_{550}) of parallel cultures of *T. thermophilus* HB8 (∗), HB8Δ*slpA* (■), HB8C27 (●), HB27 (+), HB27Δ*slpA* (□), and HB27C8 (○) at different times are shown.

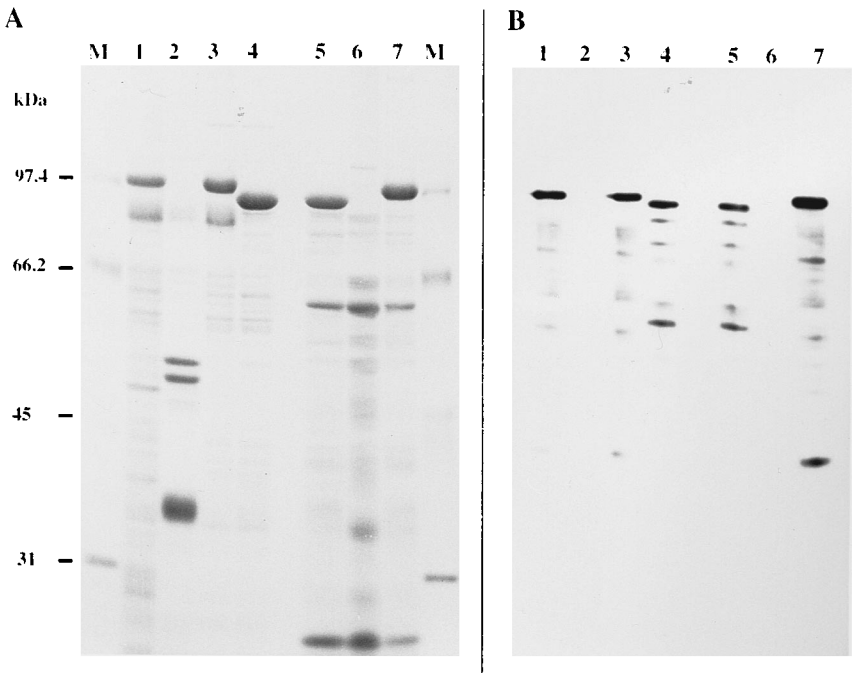

FIG. 4. Membrane protein patterns of *T. thermophilus* strains. Cell envelopes of *T. thermophilus* HB8 (lane 1), HB8Δ*slpA* (lane 2), HB8C8 (lane 3), HB8C27 (lane 4), HB27 (lane 5), HB27Δ*slpA* (lane 6), and HB27C8 (lane 7) were purified and treated for SDS-PAGE. (A) Coomassie blue staining; (B) Western blot containing a 1/20 dilution of the samples used for panel A. S-layer proteins and their fragments were detected with a mixture of six monoclonal antibodies against P100.

(4) to obtain S-layer crystals. Crystals were then negatively stained and analyzed under an electron microscope. As shown in the inset in Fig. 5c, the P95 protein obtained from cell envelopes of strain HB8C27 (Fig. 4A, lane 4) was able to form hexagonal crystals, similar in structure (P6) and cell dimensions to those built up by the P100 protein (4, 5). Thus, P95 from *T. thermophilus* HB27 forms an S-layer when expressed on the surface of *T. thermophilus* HB8.

DISCUSSION

In natural environments, bacterial cells frequently have a paracrystalline S-layer as their outermost envelope (21). However, very little is known about the actual role of these structures in most organisms, about their phylogenetic origin, or about the possibility of horizontal transference of the corresponding genes. In this article, we demonstrate that the last possibility exists, at least between related strains belonging to genus *Thermus*, one of the most thermophilic branches of domain *Bacteria* (31).

To demonstrate this, we first obtained null mutants of the S-layer gene (*slpA*) in *T. thermophilus* HB8 by gene replacement (Fig. 2). Total DNA from one of these mutants, HB8Δ*slpA*-3, was used to obtain the corresponding *slpA* mutant from *T. thermophilus* HB27, a result which allowed us to demonstrate (i) the feasibility of horizontal transference of linear DNA under strong selective pressure and (ii) the existence of enough sequence homology to allow site-directed recombination.

Addition of heterologous chromosomal DNA from wild-type strains to exponential cultures of these S-layer mutants allowed the selection after continuous growth of strains with doubling times identical to those of the respective parental strains (Fig. 3). The analysis of membrane proteins from the new isolates demonstrated that these derivatives carried a major protein whose M_r was identical to that of the S-layer monomer from the DNA donor (Fig. 4A). Subsequent immunoblotting analysis with monoclonal antibodies (Fig. 4B) showed a pattern of S-layer fragments which clearly identified these proteins as the homologous (HB8C8) or heterologous (HB8C27 and HB27C8) S-layer monomer, thus confirming the horizontal transference and expression of the corresponding S-layer genes.

Previous results from our laboratory demonstrated that the insertional inactivation of the *slpA* gene induced in *T. thermophilus* HB8 the overexpression of a group of three major proteins (15). As these proteins were detected in those experiments with polyclonal antibodies against the P100 S-layer protein, the possibility existed that they were expressed from the remaining 5′ region of *slpA*. However, the data presented in this article clearly demonstrated that these proteins were expressed from a different locus, as we detected them even in a fully *slpA* deleted strain (Fig. 2). This conclusion was further reinforced by the absence of positive identification of these bands with a mixture of six monoclonal antibodies directed against different regions of the P100 protein (Fig. 4B, lane 2).

The overexpression of these membrane proteins in *T. thermophilus* HB8 from an *slpA*-independent origin suggests that the regulatory controls which allow the cell to sense the absence of a functional S-layer on the cell envelope could be in some way similar to that described for porins (11). Furthermore, the expression of a homologous or heterologous S-layer blocked the overproduction of these proteins (Fig. 4A, lanes 3 and 4), implying that the regulatory system was recognizing the heterologous S-layer as functional.

By contrast, and despite its apparent phylogenetic relationships to strain HB8, *T. thermophilus* HB27 behaved differently. In fact, HB27Δ*slpA* did not overproduce any protein as a consequence of the inactivation of the *slpA* gene (Fig. 4A, lane 6), suggesting the existence of additional mechanisms of control or, alternatively, the absence of an active S-layer sensor. In

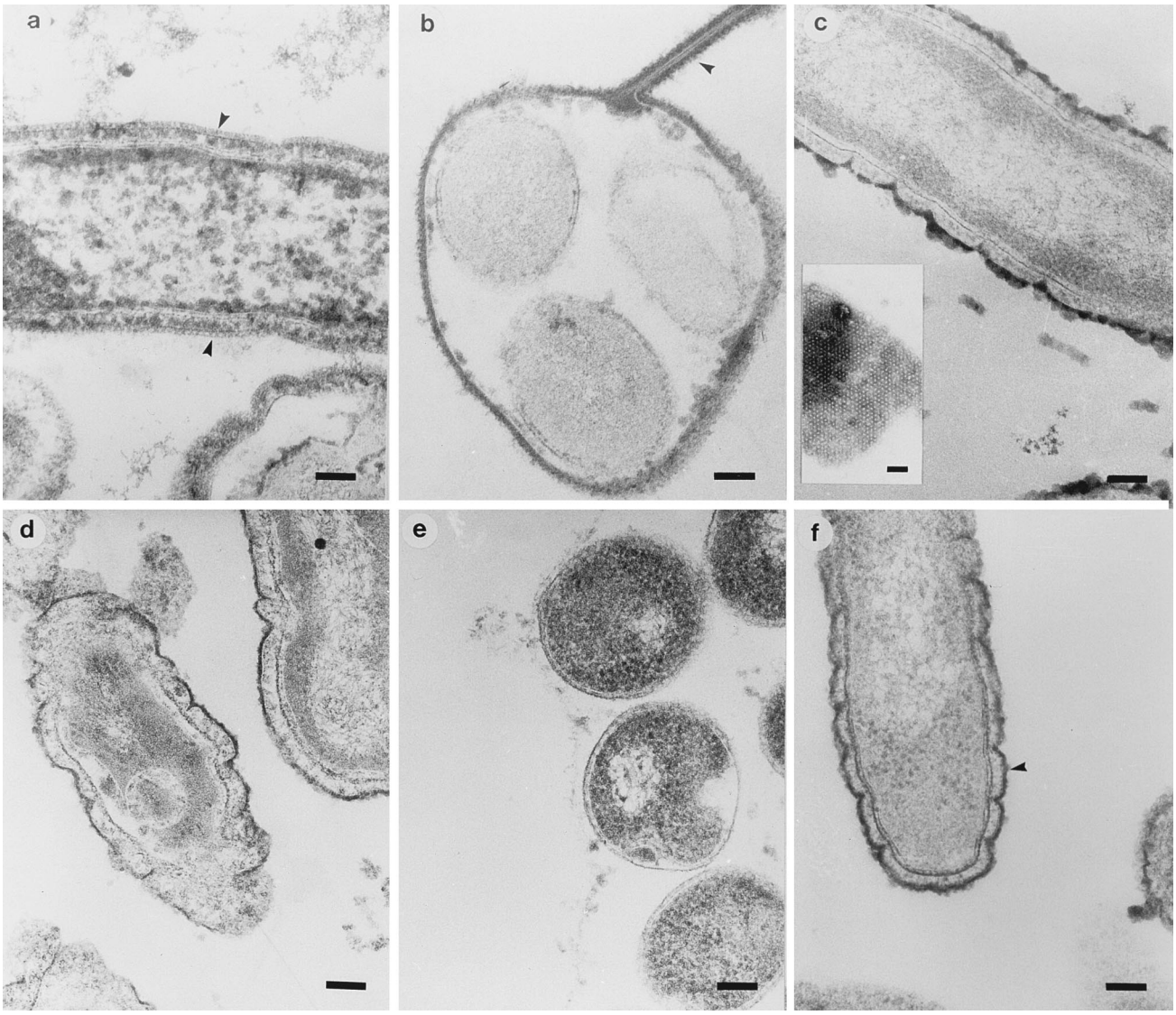

FIG. 5. Microscopic analysis of different *T. thermophilus* strains. Thin sections of *T. thermophilus* HB8 (a), HB8Δ*slpA* (b), HB8C27 (c), HB27 (d), HB27Δ*slpA* (e), and HB27C8 (f) are shown. The inset in panel c corresponds to a crystal obtained from cell envelopes of HB8C27. Arrowheads in panels a, b, and f indicate those regions in which a regular pattern could be detected. Bars represent 100 nm.

any case, these results demonstrated the existence of significant genetic differences between *T. thermophilus* HB8 and HB27.

Thin sections of whole cells revealed similar ultrastructures for HB8Δ*slpA* and HB27Δ*slpA* mutants. Both grew as groups of cells inside common envelopes (Fig. 5b and e). However, these envelopes were clearly different. While the common envelope stained poorly and did not present any regular pattern in *T. thermophilus* HB27Δ*slpA* (Fig. 5e), it appeared as a well-stained and regular single or double bilayer in *T. thermophilus* HB8Δ*slpA* (Fig. 5b, arrowhead), suggestive of a proteinaceous nature. Previous results with insertional *slpA* mutants from *T. thermophilus* HB8 demonstrated that this cell envelope copurified with an insoluble membrane fraction whose main components are the three overproduced proteins (14). Our laboratory is now involved in biochemical and genetic analyses of these proteins.

The horizontal transference of heterologous S-layer genes led to the selection of new strains (HB8C27 and HB27C8) which were indistinguishable from their wild-type parental strains at the level of growth rate (Fig. 3) or ultrastructure (Fig. 5c and f). The single difference found was apparently related to the specific expression of each of the S-layer proteins. When the P100 protein was expressed, a regular layer was detected on the cell surface by thin sections. The inability to detect such regularity when P95 was expressed (Fig. 5c and d) was probably due to masking with other cell envelope components which are known to mask the *T. thermophilus* S-layer (3), as EDTA-Triton X-100-insoluble fractions revealed the presence of hexagonal crystals on membrane fractions of HB8C27 (Fig. 5c, inset).

The possibility of horizontal transference of S-layer genes between different *Thermus* strains has thus been demonstrated. In consequence, the possibility exists that new strains, carrying a modified or even completely different S-layer, can successfully compete in natural environments, provided the existence

of selective pressures against the "old" S-layer. Phages and proteolytic enzymes are good candidates to force horizontal transference of S-layers genes in a process similar to what we have described here.

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REFERENCES

1. **Berenguer, J., M. Faraldo, and M. de Pedro.** 1988. Ca^{2+}-stabilized oligomeric protein complexes are major components of the cell envelope of *Thermus thermophilus* HB8. J. Bacteriol. **170:**2441–2447.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
3. **Castón, J., J. Carrascosa, M. de Pedro, and J. Berenguer.** 1988. Identification of a crystalline layer on the cell envelope of the thermophilic eubacterium *Thermus thermophilus*. FEMS Lett. **51:**225–230.
4. **Castón, J. R., J. Berenguer, M. A. de Pedro, and J. L. Carrascosa.** 1993. S-layer protein from *Thermus thermophilus* HB8 assembles into porin-like structures. Mol. Microbiol. **9:**65–75.
5. **Castón, J. R., J. Berenguer, E. Kocsics, and J. L. Carrascosa.** 1994. Three-dimensional structure of different aggregates built up by the S-layer protein of *Thermus thermophilus*. J. Struct. Biol. **113:**164–176.
6. **Faraldo, M. L., M. A. de Pedro, 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.
7. **Faraldo, M. L., M. A. de Pedro, and J. Berenguer.** 1991. Cloning and expression in *Escherichia coli* of the structural gene coding for the monomeric protein of the S-layer of *Thermus thermophilus* HB8. J. Bacteriol. **173:**5346–5351.
8. **Faraldo, M. L., M. A. de Pedro, and J. Berenguer.** 1992. Sequence of the S-layer gene of *Thermus thermophilus* HB8 and functionality of its promoter in *Escherichia coli*. J. Bacteriol. **174:**7458–7462.
9. **Fernández-Herrero, L. A., M. A. Badet-Denisot, B. Badet, and J. Berenguer.** 1995. *glmS* of *Thermus thermophilus* HB8: an essential gene for cell wall synthesis identified immediately upstream of the S-layer gene. Mol. Microbiol. **17:**1–12.
10. **Hanahan, D.** 1985. Techniques for transformation of *E. coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning: a practical approach. IRL Press, Oxford.
11. **Henning, U., and R. Koebnik.** 1994. Outer membrane proteins of *Escherichia coli*: mechanism of sorting and regulation of synthesis, p. 381–395. *In* J. M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier, London.
12. **Ishiguro, E. E., W. Kay, T. Ainsworth, J. B. Chamberlain, R. A. Austin, T. J. Buckey, and T. J. Trust.** 1981. Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. J. Bacteriol. **148:**333–340.
13. **Koyama, Y., T. Hoshino, N. Tomizuka, and K. Furukawa.** 1986. Genetic transformation of the extreme thermophile *Thermus thermophilus* and of other *Thermus* spp. J. Bacteriol. **166:**338–340.
14. **Lasa, I., J. R. Castón, L. A. Fernandez-Herrero, M. de Grado, G. Olabarría, J. L. Carrascosa, M. A. de Pedro, and J. Berenguer.** 1993. Phenotypic, biochemical and structural analysis of "S-layer" mutants from *Thermus thermophilus* HB8, p. 99–104. *In* M. A. de Pedro, J.-V. Höltje, and W. Löffelhardt (ed.), Bacterial growth and lysis: metabolism and structure of the bacterial sacculus. Plenum Press, New York.
15. **Lasa, I., J. R. Castón, L. A. Fernandez-Herrero, M. A. Pedro, and J. Berenguer.** 1992. Insertional mutagenesis in the extreme thermophilic eubacterium *Thermus thermophilus*. Mol. Microbiol. **11:**1555–1564.
16. **Lasa, I., M. de Grado, M. A. de Pedro, and J. Berenguer.** 1992. Development of *Thermus-Escherichia* shuttle vectors and their use for the expression in *Thermus thermophilus* of the *celA* gene from *Clostridium thermocellum*. J. Bacteriol. **174:**6424–6431.
17. **Lennox, E. X.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1:**190–206.
18. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3:**208–218.
19. **Mather, M. W., and J. A. Fee.** 1992. Development of plasmid cloning vectors for *Thermus thermophilus* HB8: expression of a heterogeneous, plasmid-borne kanamycin nucleotidyltransferase gene. Appl. Environ. Microbiol. **58:**421–425.
20. **Matsumura, M., and S. Aiba.** 1985. Screening for thermostable mutant of kanamycin nucleotidyltransferase by the use of a transformation system for a thermophile, *Bacillus stearothermophilus*. J. Biol. Chem. **260:**15298–15303.
21. **Messner, P., and U. B. Sleytr.** 1992. Crystalline bacterial surface layers. Adv. Microb. Physiol. **33:**213–275.
22. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Oshima, M., and K. Imahori.** 1974. Description of *Thermus thermophilus* (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. Int. J. Syst. Bacteriol. **24:**102–112.
24. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
26. **Sleytr, U. B., and P. Messner.** 1983. Crystalline surface layers on bacteria. Annu. Rev. Microbiol. **37:**311–339.
27. **Spratt, B. G.** 1975. Distinct penicillin binding proteins involved in the division, elongation and shape of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **72:**2999–3003.
28. **Vázquez, J. A., S. Berrón, M. O'Rourke, G. Carpenter, E. Feil, N. H. Smith, and B. G. Spratt.** 1995. Interspecies recombination in nature: a meningococcus that has acquired a gonococcal PIB protein. Mol. Microbiol. **15:**1001–1007.
29. **Vieira, J., and J. Messing.** 1987. Production of single stranded plasmid DNA. Methods Enzymol. **153:**3–11.
30. **Whatmore, A. M., V. Karpus, J. M. Musser, and M. A. Kehoe.** 1995. Molecular population genetic analysis of the enn subdivision of group A streptococcal *emm*-like genes: horizontal gene transfer and restricted variation among *enn* genes. Mol. Microbiol. **15:**1039–1048.
31. **Woese, C. R., O. Kandler, and M. L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains *Archaea*, *Bacteria*, and *Eukarya*. Proc. Natl. Acad. Sci. USA **87:**4576–4581.
32. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pU19 vectors. Gene **33:**103–119.