Extremophiles (2002) 6:225–232
Digital Object Identifier (DOI) 10.1007/s00792-001-0246-3

ORIGINAL PAPER

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The periplasmic space in *Thermus thermophilus*: evidence from a regulation-defective S-layer mutant overexpressing an alkaline phosphatase

Received: May 10, 2001 / Accepted: August 20, 2001 / Published online: February 27, 2002

Abstract The presence of a periplasmic space within the cell envelope of *Thermus thermophilus* was analyzed in a mutant (HB8ΔUTR1) defective in the regulation of its S-layer (surface crystalline layer). This mutant forms round multicellular bodies (MBs) surrounded by a common envelope as the culture approaches the stationary phase. Confocal microscopy revealed that the origin of the MBs is the progressive detachment of the external layers coupled with the accumulation of NH_2-containing material between the external envelopes and the peptidoglycan. A specific pattern of proteins was found as soluble components of the intercellular space of the MBs by a single fractionation procedure, suggesting that they are periplasmic-like components. To demonstrate this, we cloned a gene (*phoA*) from *T. thermophilus* HB8 encoding a signal peptide-wearing alkaline phosphatase (AP), and engineered it to be overexpressed in the mutant from a shuttle vector. Most of the AP activity (>80%) was found as a soluble component of the MBs' intercellular fraction. All these data indicate that *Thermus thermophilus* actually has a periplasmic space which is functionally similar to that of Proteobacteria. The potential application of the HB8ΔUTR1 mutant for the overexpression of periplasmic thermophilic proteins is discussed.

Key words Alkaline phosphatase · Overexpression · Periplasm · S-layer · *Thermus*

Communicated by G. Antranikian

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Introduction

The genus *Thermus* has been classified as one of the oldest branches of bacterial evolution in most phylogenetic trees based on 16S rRNA (Hartmann et al. 1989; Woese et al. 1990). However, recent re-interpretation of the bacterial evolution using criteria that include the ultrastructure of the cell envelopes, identify this genus as an intermediate evolutionary step between the Gram-positive (monoderms) and the Gram-negative (diderms) bacteria (Gupta 2000). Actually, the cell envelope of *Thermus* spp. is a multilayered structure that does not fit neither of the above well-known patterns (Castón 1992). As in Gram-negatives, the cytoplasmic membrane is surrounded by a thin peptidoglycan (PG) layer, which is responsible for its negative stain in the Gram reaction. However, the PG has a composition more similar to that of Gram-positives, with L-ornithine instead of m-diaminopimelic acid, and diglycine crosslinks between the tetrapeptides (Quintela et al. 1995). Overlaying the PG there are at least three additional layers. There is a thick, and apparently unstructured, layer (intermediate layer, IL), which is limited by a regular proteinaceous structure known as the S-layer (surface crystalline layer) (Castón et al. 1988; Faraldo et al. 1992). Finally, a sugar-rich surface, sensitive to ethylenediaminetetraacetic acid (EDTA), protects the S-layer from the outside (Castón et al. 1988).

At present, it is not known whether or not any of the above layers that surround the PG constitute a membrane-like permeability barrier functionally similar to the outer membrane of Proteobacteria (Nikaido 1996). The presence of a cell wall-binding domain, known as the S-layer homology (SLH) domain, at the N-terminus of the S-protein from *T. thermophilus* HB8 (Olabarría et al. 1996), a common feature among S-layer proteins from Gram-positives (Lupas et al. 1994), is an argument against the presence of such an "outer membrane" in these bacteria.

In this article we take advantage of the availability of a *T. thermophilus* HB8 mutant defective in the control of the S-layer expression (Castán et al. 2001) to unequivocally identify the presence of a membrane-like permeability barrier which limits a periplasmic hydrophilic space in this genus.

The specific overexpression of a soluble form of a thermophilic alkaline phosphatase in such a compartment confirms the Proteobacteria-like functional character of the periplasmic space of *T. thermophilus*.

Materials and methods

Bacterial strains, plasmids, and growth conditions

Thermus thermophilus HB8 (ATCC 27634) (Oshima and Imahori 1974) was obtained from the American Type Culture Collection (Rockville, Md.). *Escherichia coli* strains TG1 [*supE*, Δ(*nsdM-mcrB*) *5*(r_k-m_k-*McrB*), *thi*, Δ(*lac-proAB*), *F′*(*traD36*, *proAB*$^+$, *laqI*^{q}*ZΔM15*)] and DH5αF′ [F′, *supE44*, Δ(*lacZYA-argF*)U169, (Φ80 *lacZΔM15*), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi1*, *relA1*] (Bethesda Research Laboratories, Gaithersburg, Md.), were used as hosts for the construction of plasmids. Plasmids pUC119 (Viera and Messing 1987), pMK18 (de Grado et al. 1999), and pRCS1.KB (Fernández-Herrero et al. 1995a) were used.

T. thermophilus was grown at 70°C in liquid media (Fernández-Herrero et al. 1995b). For positive selection, 30 μg/ml of kanamycin was added to liquid medium or agar (1.5% w/v) plates. *E. coli* strains were grown at 37°C in LB medium (Lennox 1955), and selected for plasmid presence on agar plates containing ampicillin (100 μg/ml) or kanamycin (30 μg/ml).

Cloning and expression of the *phoA* gene from *T. thermophilus* HB8

Plasmid pAPA was isolated from a gene expression library of *T. thermophilus* HB8 DNA constructed in pUC119 (Fernández-Herrero et al. 1997). Around 8,000 clones were transferred to nitrocellulose filters and subjected to lysis and denaturation as described for colony blotting (Sambrook et al. 1989). The filters were incubated with the fluorogenic substrate ECL (Amersham-Pharmacia Biotech, http://www.apbiotech.com) at room temperature under the conditions described in the ECL kit. Three different plasmids were isolated from fluorescent clones and analyzed by partial sequencing. One of these clones, named pAPA, was fully sequenced by automatic methods. The sequence of the *phoA* gene described in this article has been registered under the accession number AJ309568.

For the overexpression of the *phoA* gene in the HB8ΔUTR1 mutant, the coding sequence of the gene was amplified by polymerase chain reaction (PCR) with the oligonucleotides OAP-10 (5′GGCATATGAAGCGAAGG3′) and OAP-13 (5′GGGGTCGACGGCCCAGAC3′) containing restriction sites for the enzymes *Nde*I and *Sal*I, respectively (underlined). The gene was cloned between the *Nde*I-*Sal*I sites of plasmid pRCS1KB, further isolated by *Eco*RI-*Hin*dIII digestion, and inserted into the equivalent sites of the *E. coli–Thermus* shuttle vector pMK18. In the final plasmid, named pMKPA, the *phoA* gene was expressed from the strong *PslpA* promoter in a multicopy *E. coli–Thermus* sp. shuttle plasmid.

Protein analysis

Protein profiles were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli and Favre 1973). Proteins were detected by Coomassie Blue staining. For the analysis of the soluble content of the multicellular bodies (MBs), the culture was collected by low-speed centrifugation and resuspended in Tris-ClH 20 mM (pH 7.5) buffer by repeated pipetting through a P1000 tip. Particulate and soluble fractions were separated by two sequential centrifugation steps at low speed (15,000 *g* for 15 min) to recover cells and large envelope fragments, and at high speed (60,000 *g*, 30 min) to eliminate small vesicles from the soluble fraction. To separate the cell envelope insoluble fraction from the cytoplasm, cells from the low-speed pellet were broken by sonication, and soluble and particulate fractions separated by high-speed centrifugation as above.

Electron and confocal microscopy

For electron microscopy, bacteria were treated as previously described (Castán et al. 2001). Confocal microscopy was developed in a Radiance 2000 microscope (Bio-Rad, Hercules, CA, USA). Samples were labeled with fluorescent succinimidyl ester derivatives of Texas Red, Oregon Green 488, and Oregon Green 514 (Molecular Probes, Leiden, The Netherlands; Catalog numbers T-6134, O-6147, and O-6139, respectively) or with the nucleic acid-specific dye Propidium Iodine (Pierce, Rockford, IL, USA). Under basic pH the succinimidyl ester derivatives bind to free amino groups of proteins and sugars. For the labeling, culture samples containing around 2×10^9 bacteria were collected at room temperature by low-speed centrifugation (3,000 *g*, 5 min), and carefully resuspended in 0.5 ml of 0.1 M sodium bicarbonate, using wide-bored pipettes to avoid the rupture of the MBs. A solution (5 μl) of the succinimidyl ester derivative in dimethyl sulfoxide (2 mg/ml) was added to the samples and allowed to react for 15 min at room temperature. When a second succinimidyl derivative had to be used, samples were immediately centrifuged as before, resuspended in 0.5 ml of 0.1 M sodium bicarbonate and labeled with the second dye as above. Labeling was stopped by adding 100 μl of 0.2 M Tris-ClH pH 7.5. Samples were centrifuged again and cells were resuspended in a mixture of Mounting Media (Sigma, St Louis, MO, USA) and 2% Ficoll (9 : 1 vol : vol). For the analysis of samples with large MBs, aliquots of the cell suspension were extended as thick layers using cut cover-slips (0.1 mm thick) as spacers between a regular microscope glass slide and a cover-slip. To avoid distortion of the largest MBs, samples of the cell suspension were mixed with liquefied gelatin (3% w/v). Drops of the mixture were laid onto cover slips, allowed to jellify, and mounted on regular glass slides using 2 mm spacers.

Results

The fine structure of the MBs

In the HB8ΔUTR1 mutant, the S-layer encoding gene is transcribed from a promoter which lacks the sequences required for its transcriptional and translational controls (Fernández-Herrero et al. 1997; Castán et al. 2001). As a consequence, cells are unable to coordinate the amount of external envelopes synthesized with that of the underlying layers, leading to the formation of multicellular bodies (MBs) (Castán et al. 2001). Figure 1 shows a thin section of a MB of the HB8ΔUTR1 mutant. As can be observed, sections from 14 cells on a single MB are connected by a common envelope (panel A) which is continuous with their external layers (see panel B), in such a way that the inward-looking part of each cell has a "nude" aspect, whereas the outward-facing side has a wild type-like profile.

Development of the MBs

To follow the generation of the MBs, samples of increasing age of the HB8ΔUTR1 mutant were covalently stained with Texas Red, and analyzed by confocal microscopy (Fig. 2). Filaments with small blebs containing a strongly stained material were detected early during growth of the mutant (panel A). These blebs were most often found at sites where cell division was taking place, although smaller blebs were also detected randomly distributed on the surface of the filaments (arrows in panel A). The blebs increased their size as the cells grew, and concomitantly forced them to curl and further divide inside them, while keeping a partial attachment to the surface (sections in panels B–C). In more aged cultures, most cells were already located inside MBs around 5 μm in diameter (panels D–F). Upon further incubation under mild stirring, MBs as large as 50 μm in diameter became common (panels G–I). Interestingly, cells were always attached to the MBs envelope, keeping the internal volume cell-free, irrespective of culture age and size (compare central section E with the bottom and top sections shown in panels D and F, respectively, and series G–I). Interestingly, intercellular Texas Red-stained material was apparently more diluted in large than in small MBs (compare panels A, B, E and H).

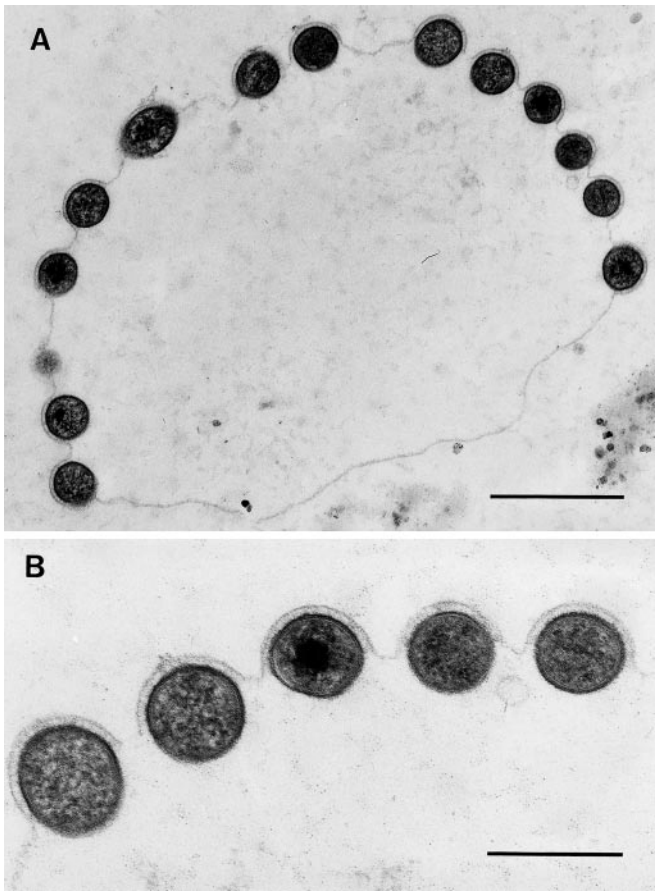

Fig. 1A,B. Thin section of a multicellular body (*MB*). **A** A thin section of a MB in which the continuity of the external envelope can be seen. **B** A detail of part of the MB. The *bars* correspond to 1.0 and 0.5 μm for panels **A** and **B**, respectively

The MBs are filled structures

The round form of the MBs in liquid cultures supports the existence of a filling material that confers enough internal osmotic pressure to keep them round and tight (Fig. 2). In addition, the results of Fig. 2 also supported the existence of NH_2-wearing compounds inside the MBs that can be stained with Texas Red. In order to discover whether the internal labeling was a specific effect of the Texas Red, we used succinimidyl derivatives of other fluorescent dyes. As shown in Fig. 3, labeling with the succinimidyl ester derivatives of Texas Red (TR), Oregon Green 488 (OG488) and Oregon Green 514 (OG514) gave strikingly different results. In this Figure, the green channel is presented on the left (A, C, E, and G) and the red one on the right (B, D, F, and H). The presence of reactive material filling the MBs was clearly revealed by OG488 (panels A and E), whereas OG514 specifically stained the external envelope of the MBs (C and G), leaving the internal material unstained. In both instances, simultaneous staining with Propidium Iodine (PI) showed the cells distributed around the inner surface of the MBs (F and H). Simultaneous labeling with OG514 and TR confirmed the integrity of the MBs, and therefore the lack of reactivity of OG514 with the intercellular material, which was clearly labeled by the TR dye (B and D). Individual cells and filaments were equally labeled by the three succinimidyl derivatives (not shown).

The MBs internal volume contains specific proteins

In an attempt to characterize the material that fills the MBs, a simple method for its isolation was developed (see Materials and methods). In essence the external membrane of the MBs was broken by repeated pipetting through a 1 ml tip, and the cells and particulate material separated from the soluble proteins by high-speed centrifugation. As shown in

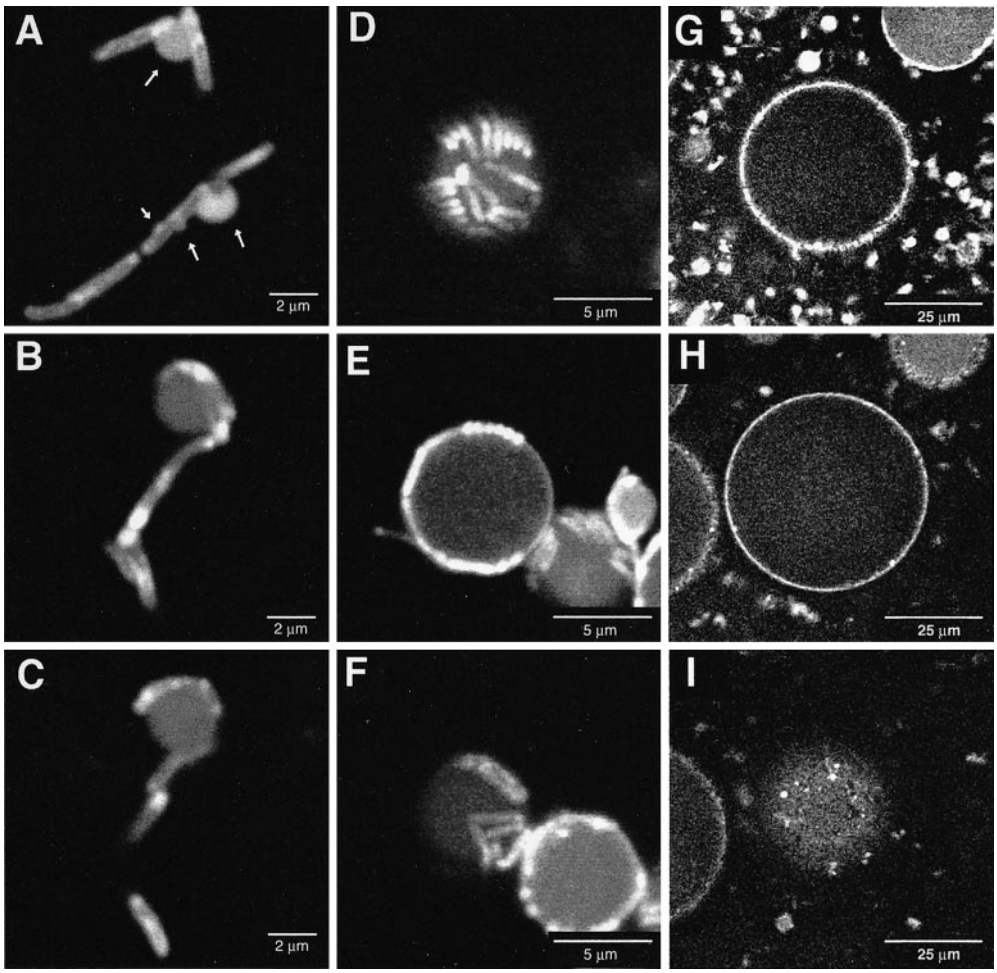

Fig. 2A–I. Development of MBs in the HB8ΔUTR1 mutant. Cells from cultures of the HB8ΔUTR1 mutant were collected at different growth times, labeled with Texas Red (TR), and section series (0.44 µm thick) analyzed by confocal microscopy. **A** Section from a sample of OD_{550} = 0.4; **B** and **C** Section series from a sample of OD_{550} = 0.6; **D**–**F** Section series from a sample of OD_{550} = 1,2; **F**–**I** Section series from a culture grown for 24 h. *Arrows in panel A* point to blebs on the cell surface

Fig. 4, several soluble proteins appeared in this fraction (lane 6), that were absent from a parallel treatment of the wild type strain (lane 3). Interestingly, the protein profile of the MBs' intercellular fraction is different from that of the cytoplasm and the membrane (compare lane 6 with lanes 4 and 5), supporting their compartment-specific nature. Parallel western blot failed to detect the abundant cytoplasmic cpn60 chaperonin in this fraction (results not shown).

Overexpression of an alkaline phosphatase in the HB8ΔUTR1 mutant

Alkaline phosphatases (AP) have been used as reporters for periplasmic location of protein fusions in Gram-negative bacteria (Derman and Beckwith 1991). We cloned in *E. coli* the gene *phoA* of *T. thermophilus* HB8 from an expression library (see Materials and methods). The sequence of the gene encoded a 501 amino-acid long protein which was 93% and 98% identical to AP described for *Thermus sp* strain FD3041 (Accession no 086025) and *T. thermophilus* GK24 (Accession no. Q9RA56), respectively. The optimum pH and temperature for the AP from *T. thermophilus* expressed in *E. coli* were 11.3 and 80°C, respectively (data not shown), with *p*-nitrophenyl phosphate as chromogenic substrate.

The sequence alignment of the three thermophilic AP proteins revealed almost identical signal sequences at their N-terminus. When expressed in *E. coli*, this signal sequence allows the location of the *T. thermophilus* HB8 AP within the periplasm (data not shown). In order to express this protein in the HB8ΔUTR1 mutant and in its parental strain up to a level at which it could easily be detected, we used the pMKPA plasmid (see Materials and methods). Plasmid pMK18 was used as control for the basal level of AP activity in both strains. As shown in Fig. 5, the AP basal level in the mutant transformed with plasmid pMK18 was so low that no signal could be detected when a fluorogenic substrate was added (Fig. 5, panel B). By contrast, an intense fluorescent signal was associated with the MBs when plasmid pMKPA was used (Fig. 5, panel D). Longer exposure times allowed the detection of fluorescence associated with individual cells in the mutant and wild type strains transformed with pMKPA (data not shown).

The above data demonstrate that most of the AP activity was associated with the MBs, suggesting a relationship between the volume of the MBs and their fluorescence intensity. However, the data did not allow us to test whether the activity was associated with the cells or soluble inside the MBs' intercellular space. To check this, we used the fractionation procedure described above, and measured the

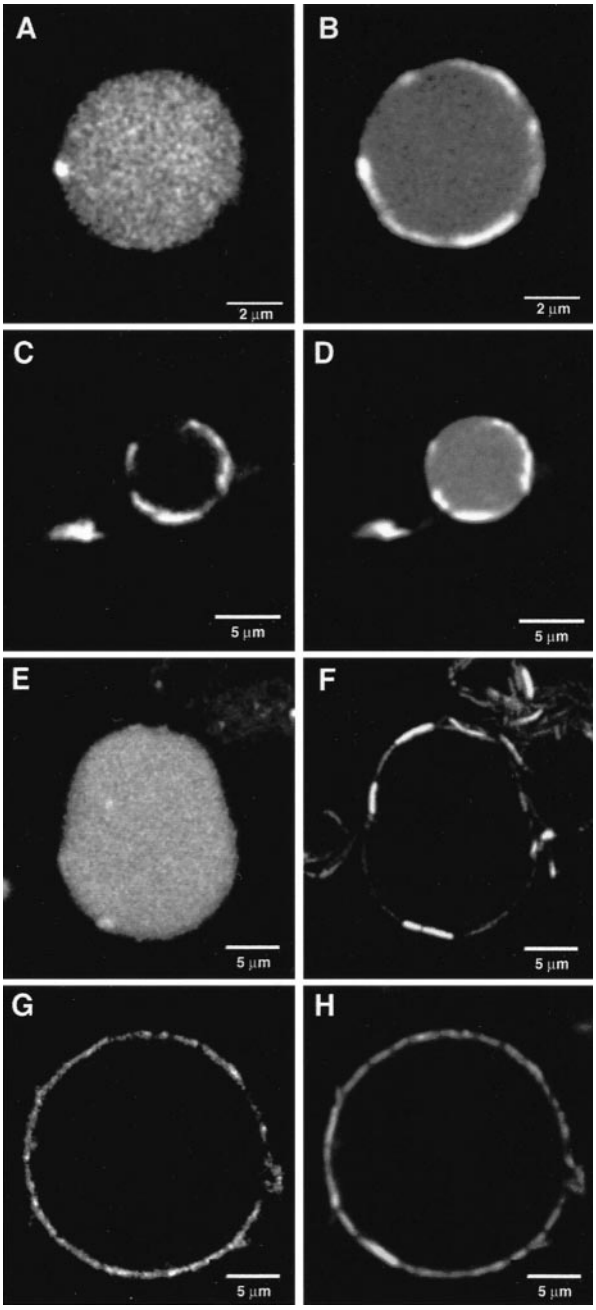

Fig. 3A–H. Differential labeling of MBs with fluorescent dyes. Pairs of images are shown corresponding to double-labeling experiments of sections of MBs from growing cultures of the HB8ΔUTR1 mutant, with the green channel *on the left* (**A**, **C**, **E**, **G**) and the red one *on the right* (**B**, **D**, **F**, **H**). Images: **A**–**B** OG488 and TR; **C**–**D** OG514 and TR; **E**–**F** OG488 and Propidium Iodine (PI); **G**–**H** OG514 and PI

amount of AP activity corresponding to the MBs' intercellular fraction and that associated with the cells. As shown in Table 1, the HB8ΔUTR1 mutant transformed with pMKPA expressed around ten times more activity than the wild type in the same conditions, and about 200-fold with respect to the basal AP level in cells transformed with pMK18. Indeed, most of this activity (>80%) appeared in a soluble form in the fraction corresponding to the MBs content.

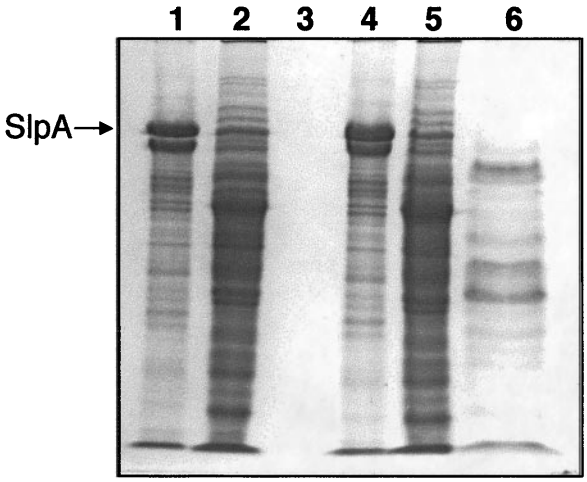

Fig. 4. A specific protein profile is found in the intercellular space of the MBs. Cells from *Thermus thermophilus* HB8 and its HB8ΔUTR1 derivative were grown under slow stirring up to OD_{550} of 0.8, and fractions corresponding to the insoluble (*1*, *4*), soluble (*2*, *5*), and intercellular content (*3*, *6*) were separated and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The position of the 100-kDa SlpA protein is labeled with *an arrow*. Note that no protein could be extracted from the wild type cells after pipetting through a 1 ml tip (*lane 3*)

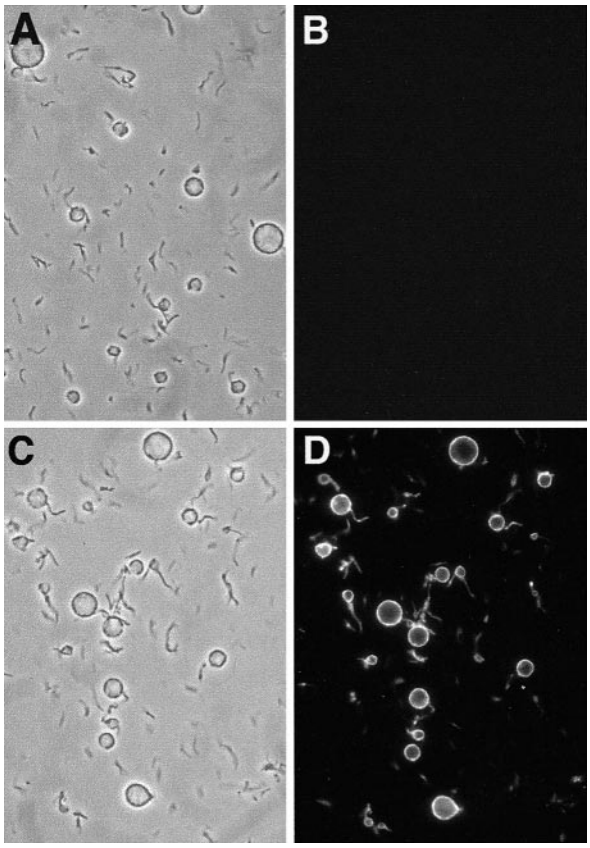

Fig. 5A–D. In situ detection of the alkaline phosphatase (AP) overexpressed in the HB8ΔUTR1 mutant. Parallel cultures of the HB8ΔUTR1 mutant transformed with pMK18 (**A**, **B**) or pMKPA (**C**, **D**) were grown up to an OD_{550} of 0.8 and immediately incubated at room temperature in the presence of the ECF fluorogenic substrate. The images correspond to phase contrast (**A**, **C**) and fluorescence (**B**, **D**)

Table 1. Distribution of alkaline phosphatase (AP) activity in the wild type and the HB8ΔUTR1 mutant transformed with plasmid pMKPA

	Total activity[a]	Soluble (%)	Cell-associated[b] (%)
HB8	5 ± 0.5	–	100
HB8ΔUTR1	42 ± 4	82	18

[a] The AP activity was measured as described in Brickman and Beckwith (1975). The basal AP activity was around 0.2 units when both strains were transformed with pMK18
[b] This fraction corresponds to entire cells

Discussion

The interaction existing between the cell wall and the S-layer has questioned the putative presence of a outer membrane-like permeability barrier in *Thermus thermophilus* (Olabarría et al. 1996). Here we show that such a permeability barrier indeed exists and constitutes the external boundary of a hydrophilic periplasmic space which is functionally equivalent to that of Proteobacteria.

The synthesis of the S-protein in the HB8ΔUTR1 mutant does not respond to the overlapping transcriptional and translational controls described for the wild type strain (Fernández-Herrero et al. 1997), in such a way that its transcription is increased and continues for much longer than in the parental strain (Castán et al. 2001). As a consequence, the coordination between the synthesis of the different layers of the envelope is lost, leading to the formation of MBs. As shown in the series sections of Fig. 2, the MBs originate as small blebs, most of them located at the end of the cells or at division sites. We propose that very small blebs are randomly distributed along the surface of the mutant (i. e., two small blebs are seen in Fig. 2A), but they grow enough to generate a MB only at division sites, where the putative binding sites on the PG retreat inwards to form the septum. A second factor that could contribute to this effect is the accumulation of NH_2-containing material on the precursor blebs, blocking any putative re-attachment to the PG surface. Moreover, it is also possible that enzymes present in these blebs could contribute to the partial or total hydrolysis of the attachment sites. Whatever the case, the new subunits of the S-protein integrate on the bleb's surface, probably at the wild type side of the cells, leading to the formation of huge MBs.

As a consequence of the above process, the space between the cells inside the MBs is continuous with the space that separates the PG and the external envelopes of the outward-facing side (similar to the wild type) (Fig. 1). Therefore, based on this morphological point of view, the internal volume of the MBs could be envisioned as a hypertrophic equivalent of a periplasmic space.

A second conclusion that can be drawn from the results shown in Figs. 2 and 3 is the existence of a permeability barrier as part of the MBs' external envelope. This can be deduced from two observations. First, even the largest MBs are completely round in hypotonic media, supporting the presence of osmotically active compounds filling their internal space. The second argument in this sense is the differential permeability to stains as similar as OG488 and OG514 (Fig. 3). Whereas the first stained the contents of the MBs just as the TR did, the second did not penetrate the MBs' envelope. Interestingly, both dyes are rather similar molecules, the major difference being the presence of three additional fluorine atoms in OG514. We propose that this difference gives the OG5145 a more hydrophilic character which is the responsible for a lower diffusion rate through an outer membrane-like permeability barrier.

One of the most important evolutive advantages of the periplasmic space is the possibility of concentrating specific proteins within a separate cell compartment. In *E. coli*, the periplasmic space may contain up to 4% of the total proteins of the cell, most of them absent from any other cell compartment (Oliver 1996). In agreement with this function, the results from Fig. 4 show a specific pattern of soluble proteins as components of the MBs' intercellular space. Unfortunately, we could not confirm such specificity by N-terminal sequencing of two of the major protein bands of this fraction because they did not show significant similarity to any other protein from the gene bank (results not shown). Therefore, we searched for a putative "periplasmic marker" such as an alkaline phosphatase to check this.

In addition to its impressive resistance to alkali treatment and thermal denaturation, the method through which we cloned the *phoA* gene demonstrated its functionality in *E. coli*. In the mesophile, the *T. thermophilus* AP is secreted through the membrane (not shown), showing that the signal peptide sequence found at its N-terminus is also functional in the mesophile. In an earlier work, Park et al. (1999) showed that the 98% identical AP from *T. thermophilus* GK24 appeared as a soluble protein after the breakage of the cells, and proposed the sequence $G_{26}A–L_{28}$ as the processing site for a signal peptidase. However, the putative periplasmic nature of the AP was not checked in that work, probably because the protocols for the extraction of periplasmic proteins which are commonly applied in *E. coli* did not work in *T. thermophilus* GK24, as it is the case for *T. thermophilus* (not shown). By contrast, the use of the HB8ΔUTR1 mutant provided a simple method for separating putative periplasmic components from entire cells and insoluble envelopes. Using this method, most of the AP activity appeared soluble in this fraction, even after a 200-fold overproduction of the activity. Moreover, due to the fractionation method used, it is very probable that the activity that remained associated with the cells corresponds to periplasmic AP from unbroken small vesicles and individual cells.

In conclusion, our data support the existence of a periplasmic compartment in *Thermus thermophilus,* limited by a hydrophobic barrier ("outer membrane") in which specific proteins are accumulated. The composition of such an "outer membrane" (OM) is not known yet, but it is clearly associated with the S-layer (Fig. 1). However, it is unlikely that the S-layer could constitute such a permeability barrier by itself, as its 3D structure (Castón et al. 1994) revealed the presence of pores larger than those of the outer membrane porins from *E. coli* (Nikaido 1996). In fact, the S-layer does not block the access to the peptidoglycan of proteins such as

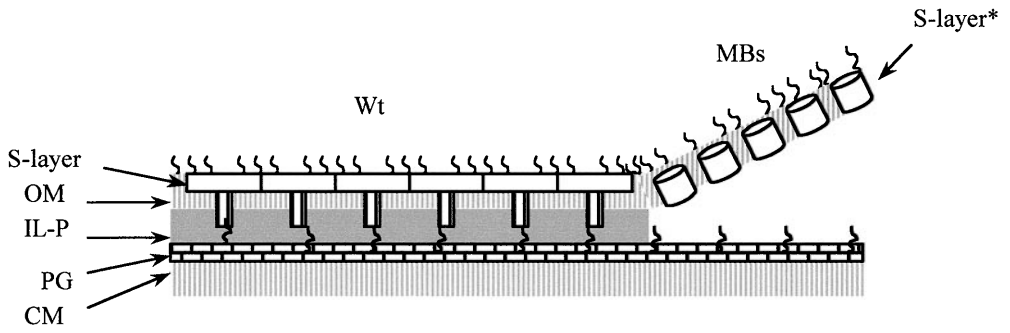

Fig. 6. The envelope profile of *T. thermophilus* showing the layered profiles of the wild type (*Wt*) and nude (*MBs*) sides of the cell envelope in the HB8ΔUTR1 mutant. In the Wt profile, the hexagonally arranged surface crystalline layer (*S-layer*) interacts through the S-layer homology (SLH) domain with the peptidoglycan (*PG*). The S-layer acts as a scaffold structure for a hydrophobic barrier (*OM*), whose sugar-rich external surface is sensitive to ethylenediaminetetraacetic acid (EDTA) and protects the S-layer. The space between the PG and the OM is a dense and gel-like hydrophilic compartment (*IL-P*), functionally equivalent to the periplasm of Proteobacteria. When the S-layer and the OM components are oversynthesized, the number of SLH binding sites is insufficient, and the horizontal interactions between the S-protein subunits pull out and detach the outer layers from the PG. Such a detachment results in a conformational change in the S-protein leading to a porin-like structure (*S-layer**)

the lysozyme (~14 kDa) in EDTA-treated cells (Castón et al. 1988). Consequently, we propose a model of cell envelope for *T. thermophilus* (Fig. 6) to explain how the MBs are formed upon the overproduction of the S-protein. In this model the S-layer could function as a scaffold for the OM, an assumption supported by the porin-like characteristics of this S-layer protein (Engelhardt and Peters 1998). When the S-protein synthesis overcomes the corresponding binding sites on the cell wall, the S-layer-OM envelope detaches, and the periplasmic components of the intermediate layer (IL-P) diffuse to the increasingly larger periplasm. It is possible that, upon detachment, a conformational change occurs in the S-layer toward a porin-like 2D structure, as happens in vitro (Castón et al. 1993). In this model, the sugar-rich and EDTA-extractable material which covers the S-layer (Castón et al. 1988) is somehow reminiscent of the lipopolysaccharide layer from Proteobacteria, and could be part of this barrier.

A final point, which opens up some very interesting avenues for applicative potential, is the possibility of using these mutants for the overproduction and purification of periplasm-directed proteins. As shown in Table 1, the amount of AP synthesized in the mutant is ~ten times higher than in the wild type, supporting the idea that a limiting synthesis or secretion step has been bypassed in the mutant. Future work will confirm whether this is a specific effect depending on the system used (AP, *PslpA* promoter) or a general one that could be extended to other periplasmic proteins of biotechnological interest.

Acknowledgments This work has been supported by projects nos BIO98–0183 from the Comisión Interministerial de Ciencia y Tecnología (CICYT) and 2FD97–0127-C02–01 co-funded by the European Union and the Spanish Ministerio de Educación y Cultura. An institutional grant from Fundación Ramón Areces is also acknowledged. P. Castán is the holder of a FPI fellowship from the Ministerio de Educación, Cultura y Deportes. O. Zafra and F. Cava are fellowship-holders from Comunidad de Madrid. E. Caro holds a fellowship from Agencia Española de Cooperación Internacional.

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