

Localized synthesis of the outer envelope from *Thermus thermophilus*

Federico Acosta · Laura Alvarez · Miguel Angel de Pedro · José Berenguer

Received: 29 September 2011 / Accepted: 13 December 2011 / Published online: 3 January 2012
© Springer 2011

Abstract In agreement with its distinct phylogenetic origin, the envelope of *Thermus thermophilus* consists of a complex pattern of layers with properties intermediate between those of Gram positives and Proteobacteria. Its cell wall of Gram positive composition is surrounded by an outer envelope that includes a crystalline layer scaffold built up by the SlpA protein, lipids and polysaccharides. The synthesis of this outer envelope has been studied by confocal microscopy. Available amino groups from the cell surface, mainly belonging to the SlpA protein, were covalently labelled in vivo with fluorescent dyes. Stained cells were able to grow without any apparent loss of viability, allowing the localization of the regions of new synthesis as dark nonfluorescent spots. Our results demonstrate that the outer envelope of *T. thermophilus* is synthesized from a central point in the cells, likely following a helical pattern. Cell poles and subpolar regions are basically inert and retain their label for generations.

Keywords *Thermus* · Outer membrane · S-layer · Growth · Thermophile

Communicated by S. Albers.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-011-0427-7) contains supplementary material, which is available to authorized users.

F. Acosta · L. Alvarez · M. A. de Pedro · J. Berenguer (✉)
Centro de Biología Molecular Severo Ochoa,
Consejo Superior de Investigaciones Científicas,
Universidad Autónoma de Madrid,
28049 Madrid, Spain
e-mail: jberenguer@cbm.uam.es

Introduction

Several extreme thermophiles and hyperthermophiles contain a regular protein array on their surface built up by the polymerization of a single (glyco)protein (Messner et al. 2008; Sara and Sleytr 2000). The roles of such structures, commonly known as S-layers (from surface layers), differ from one organism to another, but in the short phylogenetic branches typical of hyperthermophiles, they most likely constitute a way to reinforce the structure of the envelope at high temperatures and to create something similar to a periplasmic space (Beveridge et al. 1997). Other functions such as protection against predation or enzymes seem further adaptations to lower temperatures.

There are only a few studies on the incorporation of new protein subunits to the preformed S-layer, generally using fluorescent antibody techniques, protein A-colloidal gold labelling, or directly electron microscopy. In Gram positives, studies with *Bacillus stearothermophilus* showed that newly synthesized S-layer subunits were incorporated as helical bands, mainly at the septal regions. Neither shedding of intact S-layer nor turnover of individual subunits was detected (Gruber and Sleytr 1988). In *Bacillus sphaericus*, a similar septal incorporation was described, but with the new subunits incorporated as perpendicular bands with respect to the longitudinal axis of the cell, in agreement with the tetragonal lattice of its S-layer (Howard et al. 1982). In *Clostridium thermohydrosulfuricum* and *C. thermosaccharolyticum*, the incorporation of new subunits was also shown to happen at the site of division, including the accumulation of an excess of S-layer material at the site of division that can detach from the cell (Sleytr and Glauert 1976). In *Bacillus anthracis*, two S-proteins (Sap and EA1) are present simultaneously at the cell surface in different ratios depending on the growth phase.

At the late exponential growth phase, Sap covers the entire surface of the bacterium, whereas EA1 is limited to defined spots. The opposite situation is observed in the stationary phase, since EA1 appears in a series of patches that spread to form a continuous network at the cell surface, triggering the release of Sap into the culture medium (Mignot et al. 2002).

In the Gram-negative *Caulobacter crescentus*, the pattern of S-layer biosynthesis functionally delineates three regions on the cell surface. On the cell body, the S-layer grows by diffuse intercalation, resulting in a mixing of new and old material over the entire surface. In contrast, S-protein is deposited on the stalk and at the septum as entirely new material (Smit and Agabian 1982).

The genus *Thermus* forms with *Deinococcus* an independent phylum of the Bacteria Domain (Griffiths and Gupta 2007). The evolutive position of this genus as defined by its 16S rDNA sequence suggests an ancestral origin close to other phyla that include extreme thermophiles and hyperthermophiles. However, the analysis of indels and the complexity of its cell envelope suggest a phylogenetic position somehow intermediate between Gram positives and Gram negatives (Proteobacteria) (Cava et al. 2009). As in Gram positives, *Thermus* spp. has ornithine instead of diamino pimelic acid as dicarboxylic amino acid in the third position of its mureopeptides, and the bridge between such mureopeptides includes two residues of glycine (Quintela et al. 1995). Also as in Gram positives, one or more types of cell wall-associated polysaccharides are bound covalently to the peptidoglycan. In contrast, other properties are closer to Gram negatives. For example, the amount of peptidoglycan and its cross-linking degree are more similar to those of Proteobacteria, to the point that the cells do not stain with the Gram reagent. Moreover, an outer envelope (OE) surrounds the cells and generates a true periplasmic space. The nature of this OE is not yet fully understood, but it includes lipidic components, polysaccharides and proteins (Castan et al. 2002). One of the most abundant proteins of this OE is SlpA, a protein that forms a hexagonal array over the whole cell, which is thus the building block of a special kind of S-layer of hexagonal symmetry that could be better defined as a regular outer membrane layer (Engelhardt and Peters 1998). SlpA anchors the envelope to the cell wall-associated polysaccharides through a single N-terminal SLH (S-layer homology) domain.

SlpA shows intermediate characteristics between typical S-proteins and porins (Engelhardt and Peters 1998). For example, SlpA solubilization requires the presence of detergents; it contains a single SLH domain and forms structures in vitro similar to those of the OmpA protein of *E. coli* (Caston et al. 1993). In contrast, most S-layer proteins are soluble in water, form structures similar to the

native S-layer and present more than one SLH motifs in Gram positives. Actually, the synthesis of SlpA seems more tightly regulated than that of other S-layers. At the transcriptional level, proteins such as SlrA and SlpM bind to the *slpA* promoter, and at the translational level SlpA is able to bind to its own mRNA (Fernandez-Herrero et al. 1997). A final observation regarding the relevance of SlpA for the cell is that *slpA* deletion mutants present severe growth defects and morphology changes even at the level of the peptidoglycan sacculi (Cava et al. 2004). In contrast, S-proteins from Gram positives and Proteobacteria are frequently lost during growth under laboratory conditions without any noticeable change in the fitness of the cell.

In this article, we analyse the incorporation of SlpA to the OE. Because application of in vivo immunolabelling techniques was impossible at the growth temperature required for these bacteria, we developed a method based on the in vivo labelling of the OE proteins with thermo-stable fluorescent dyes that allowed us to identify the newly synthesized regions of the surface generated during cell growth. Our data point out that the synthesis of the OE of *T. thermophilus* takes place in helical bands at a central region of the cell, with full conservation of the proteins at the cell poles.

Materials and methods

Strains and growth conditions

The wild-type strain *Thermus thermophilus* NAR1 and its $\Delta slpA::kat$ derivative (Fernandez-Herrero et al. 1995) were routinely grown at 70°C in rich medium (TB) (Ramirez-Arcos et al. 1998) under strong aeration. For growth on plates, the same medium with agar (1.5% w/v) was used, and incubation was carried out in a water-saturated atmosphere for 24–48 h.

Fluorescent labelling and protein analysis

Aliquots of 1 ml of cells from exponential cultures of the wild-type and the $\Delta slpA::kat$ mutant strains were collected in early exponential phase by centrifugation (4000g, 5 min). After washing in 1 ml of phosphate saline buffer (PBS; NaCl 150 mM, KCl 2.5 mM, Na₂HPO₄ 8 mM, KH₂PO₄ 1.5 mM, pH 7.5), cells were resuspended in 1 ml of PBS and incubated for 20 min at 70°C in the presence of 6 µl (5 mg/ml in dimethyl sulfoxide) of NH₂-specific reagent succinimidyl ester of Oregon Green (Molecular Probes Europe BV, Leiden, The Netherlands). Labelling reaction was stopped by the addition of cold (4°C) Tris–ClH buffer, pH 8, to reach a final concentration of 100 mM. Cells were washed by centrifugation and

resuspended in 0.3 ml of PBS, before being broken by sonication (3 pulses, 30 s, maximum amplitude in a “Labsonic” sonicator, Sartorius) in the presence of a cocktail of protease inhibitors (“complete”, Roche). Soluble and particulate fractions were separated by centrifugation (20000g, 20 min) and insoluble fraction was further resuspended and incubated for 30 min at 37°C with 0.3 ml of Triton X100. The detergent-soluble fraction, corresponding to inner membrane proteins and some outer membrane proteins, and insoluble fraction, containing SlpA, were separated by centrifugation (20000g, 20 min). Proteins from all the fractions were denatured by boiling in Laemmli denaturation buffer and analysed by SDS-PAGE (Laemmli and Favre 1973). Detection was carried out either by Coomassie blue staining, fluorescence (Typhoon 9410 variable mode imager, Amersham) or Western blot. For Western blot, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and subjected to incubation with a mix of monoclonal mouse antibodies directed against SlpA sequence (Faraldo et al. 1991) epitopes located between amino acid positions 1–98 (3CG10), 288–556 (3ED10) and 728–917 (2AD10) (Caston et al. 1996). After washing, the presence of the antibodies bound to the membrane was detected with rabbit antimouse antibodies through a bioluminescence assay (ECL, Amersham International).

Growth of labelled cells

Cells (20 ml) from cultures incubated aerobically to log phase were collected, concentrated to 1 ml in preheated PBS by mild centrifugation, and labelled for 20 min at 70°C as above with succinimidyl esters of Texas Red or Oregon Green (Molecular Probes Europe BV, Leiden, The Netherlands). Labelled cells were harvested by mild centrifugation (2000 rpm, Hettich Zentrifugen Mikro, 5 min, 70°C) and washed two times with PBS (NaCl 150 mM, KCl 2.5 mM, Na₂HPO₄ 8 mM, KH₂PO₄ 1.5 mM, pH 7.5) before being reinoculated in 20 ml of preheated TB. For microscopy observation, samples were taken at different times and treated with formaldehyde 0.1% (v/v) before being analysed by fluorescent or confocal microscopy.

Image capture and processing

Labelled cells were directly spread onto microscope slides and left to dry at room temperature before mounting with Mowiol 4-88 Reagent (Calbiochem no. ref. 475904). Fluorescent microscopy was performed in a Zeiss Meta 510 confocal microscope. Z-stacks were obtained using a Zeiss 63 × 1.4 NA objective lens and parameters appropriate to comply with the Nyquist criterion for image sampling. Images were subjected to linear deconvolution using the

Huygens System 2.2 software (Scientific Volume Imaging B.V., Hilversum, The Netherlands). Adobe Photoshop and Image J (Wayne Rasband, NIH, USA) were used for final assembly of the images and 3D reconstructions.

Results

Fluorescent derivatives preferentially label SlpA in vivo

Covalent binding of succinimidyl ester derivatives of fluorophores to cells was very faint when cell reaction was carried out directly in TB due to the presence of free amino acids and peptides in the rich medium (not shown). However, when labelling was carried out in NH₂-free buffers like PBS, cells were uniformly labelled (see below). In order to define what components of the cells were labelled with succinimidyl ester of Oregon Green in the process, we analysed different cell fractions. As shown in Fig. 1, most of the fluorescent labelling concentrated on a major protein from the particulate cell fraction of the wild-type strain (lane 2). Other proteins, both from the soluble and the particulate cell fractions, were also labelled with lesser intensity. In comparison, a culture of a *ΔslpA::kat* derivative subjected to the same treatment showed an almost negligible labelling (lanes 1–4), supporting that most proteins were not accessible to the fluorescent derivative in this strain. A parallel Western blot carried out with a mix of three monoclonal antibodies directed against N-terminal, central and C-terminal sequences of the SlpA protein, respectively, confirmed the nature of the highly labelled membrane protein as SlpA (Fig. 1c). Also, a treatment of the particulate fraction with TX100, a detergent that solubilizes all the cytoplasmic membrane proteins and most of those of the outer membrane, revealed that most labelling was associated with the outer membrane component SlpA, or with a smaller protein band that likely represented a degradation product of SlpA because protein bands of the same size are identified by anti-SlpA monoclonal antibodies (lanes 4). All these data confirm that the fluorescent labelling of the *T. thermophilus* envelope concentrates preferentially on the SlpA protein.

Labelling is stable and uniform in wild-type cells

To follow the evolution of the labelling along the growth of *T. thermophilus*, it was necessary to confirm the stability of the fluorescence bound to the cells. For this, growing cells were labelled and washed as described in “Materials and methods” before being diluted and incubated at 70°C in preheated TB. The fluorescence associated with the cells was analysed at different times and compared with the

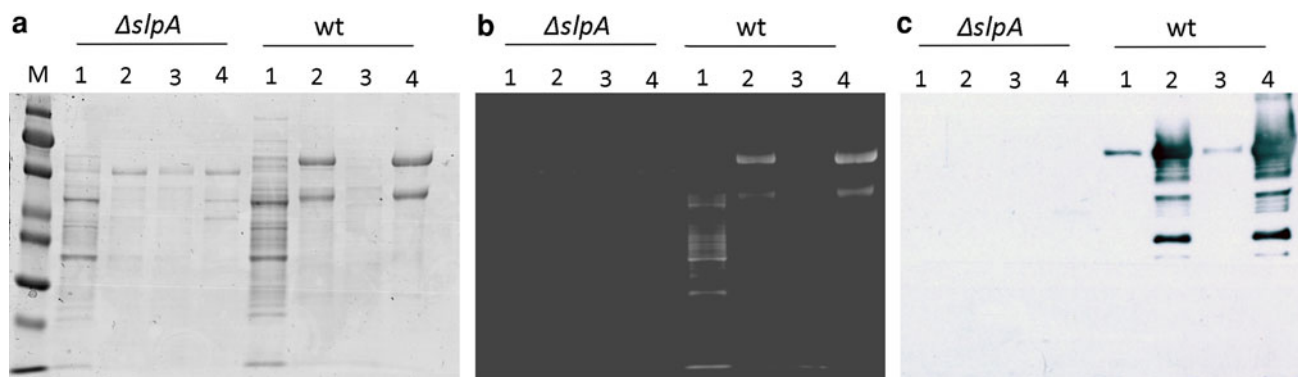


Fig. 1 Fluorescence labels preferentially to the SlpA protein. Cultures of *T. thermophilus* $\Delta slpA$ and parental (wt) strains were centrifuged and labelled in PBS with succinimidyl ester of Oregon Green as described in “Materials and methods”. Cells were disrupted, and soluble (1) and particulate (2) fractions were separated. Particulate fraction 2 was incubated with TX100 and the corresponding

soluble (3) and insoluble (4) fractions were analysed by SDS-PAGE. **a** Proteins stained with Coomassie blue. **b** Fluorescence detected. **c** Western blot with a mix of three mouse monoclonal antibodies directed against epitopes located within the N-terminal (aas 1–98), the central (aas 288–556) and the C-terminal (aas 728–917) sequence of SlpA. *M* markers (kDa): 177, 118, 75, 51, 39, 26, 18, 13

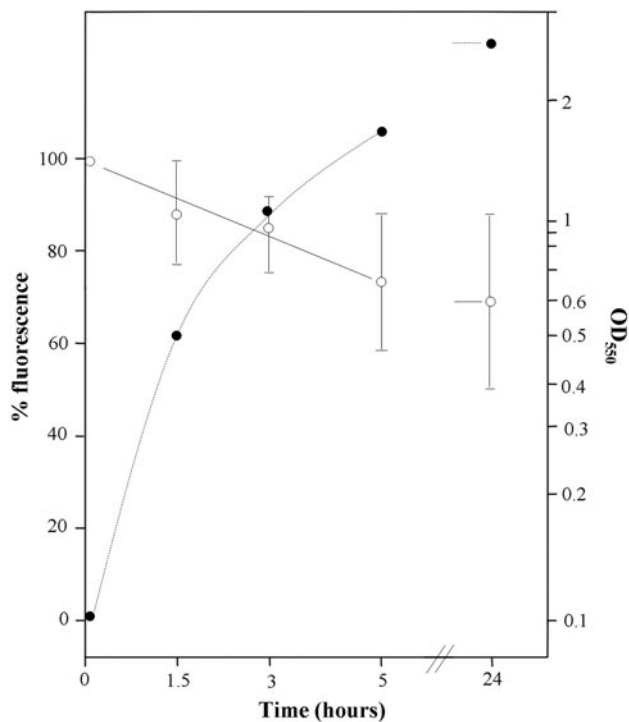


Fig. 2 Stability of fluorescent labelling in *T. thermophilus*. Figure shows the OD₅₅₀ (dotted line, black circles) and the fluorescence (solid line, white circles) bound to the cells after the indicated period of growth at 70°C of *T. thermophilus* NAR1 cultures labelled at time 0

values at the start of the experiment. The data (Fig. 2) revealed that cells grown for 24 h under these conditions still kept around 70% of the original value. These experiments also showed that cells recovered well after the covalent labelling, as they re-started growth at their normal rate with little or no lag. In consequence, we concluded that the labelling procedure was innocuous to the cells and the

signal stable enough for use to identify the regions of new synthesis on the surface of growing cells.

Homogeneity was another condition required for the use of this labelling procedure to follow the regions with newly incorporated materials. As shown in Fig. 3a, labelling of the cells resulted in uniform fluorescence that, once processed by deconvolution, was revealed as mainly associated to the cell envelope (Fig. 3b), in agreement with the concentration of the labelling on the SlpA protein shown in Fig. 1.

S-layer growth takes place at a central region of the cell

When labelled cells were allowed to grow for about one generation (50 min), dividing cells appeared strongly labelled at the cell poles (Fig. 3c), whereas the central regions showed a much lower fluorescence. Such a pattern indicates that the synthesis of the envelope and particularly, of the surface layer that concentrates most of the labelling, is conservative. Transition from the labelled to the dark sections was quite steep, as shown in the intensity analysis of Fig. 3d, thus supporting that the envelope at the cell poles and subpolar regions are preserved during the growth. A statistical series of measures from cells grown for one-and-a-half generations revealed that the measured size of the dark region was in concordance with the theoretical calculations following a fully conservative mechanism of longitudinal growth for cells of identical diameter (Fig. 3e). The deviation observed could be likely due to the optical effect produced by the fluorescence that leads to an overestimation of the labelled zones. Therefore, the cell envelope of *Thermus thermophilus* follows a conservative mechanism of growth.

To confirm this, we followed the growth of labelled cells for longer times and checked the localization of the

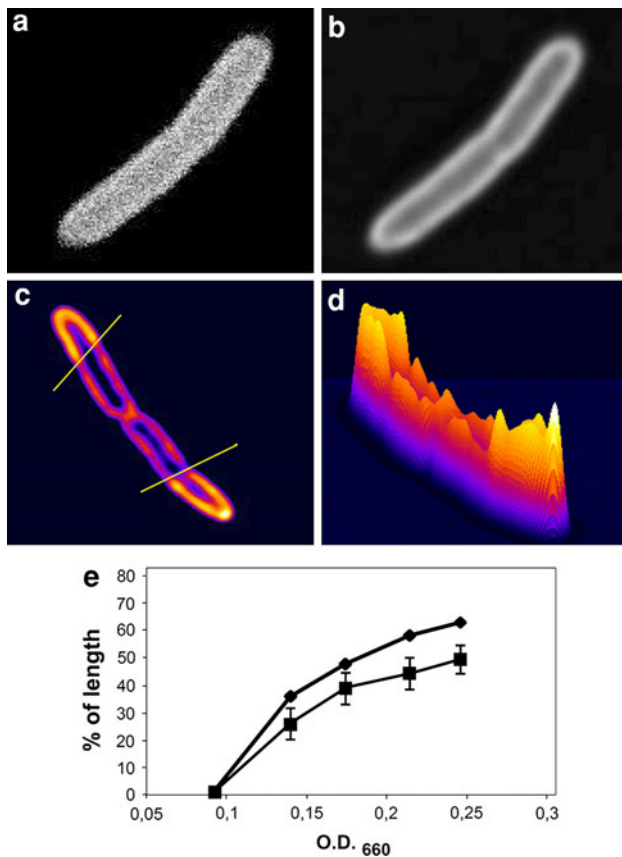


Fig. 3 Conservative growth of the external envelope in *T. thermophilus*. Confocal image from a labelled bacterium of *T. thermophilus*, before (a) and after (b) deconvolution, respectively. c Image of a bacterium corresponding to 50 min after labelling. The lines label the tilt of the transition zones with respect to the main axis of the cell. d Intensity profile of image c. e Statistics of the length of the percentage of unlabelled region (*squares*) with respect to the theoretical length (*diamonds*) for a single internal growth region along one generation growth after labelling. Each point represents around 20 individual measurements

labelling. As shown in Fig. 4, some of the cells (40% after 2.2 generations as estimated from around 100 images) on such cultures containing a single well-labelled cell pole were detected, supporting the above conclusion. Noteworthy was that many of the images, in the transitions from labelled regions to unlabelled zones, formed tilt angles with respect to the main axis of the cells (labelled as lines in Fig. 3c). A statistical analysis of more than 100 selected images supports an angle close to 60° with respect to the longitudinal axis of the cell.

Once a conservative mechanism for the growth of the cell envelope was defined, we wondered if the incorporation of new OE would take place at the old-to-new interfaces, or at a fixed central position around the future division site. In the first case, two biosynthetic regions should be active in each cell at any time, whereas in the second instance only one biosynthetically active site per

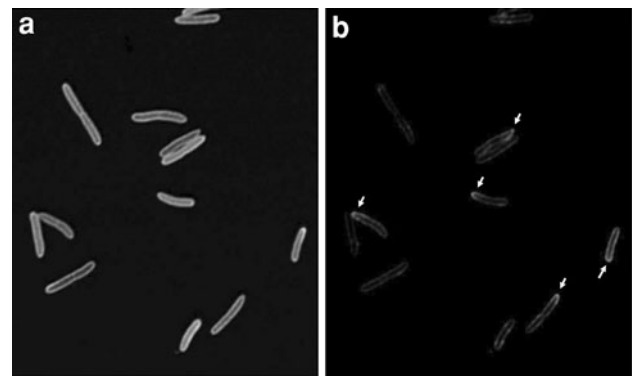


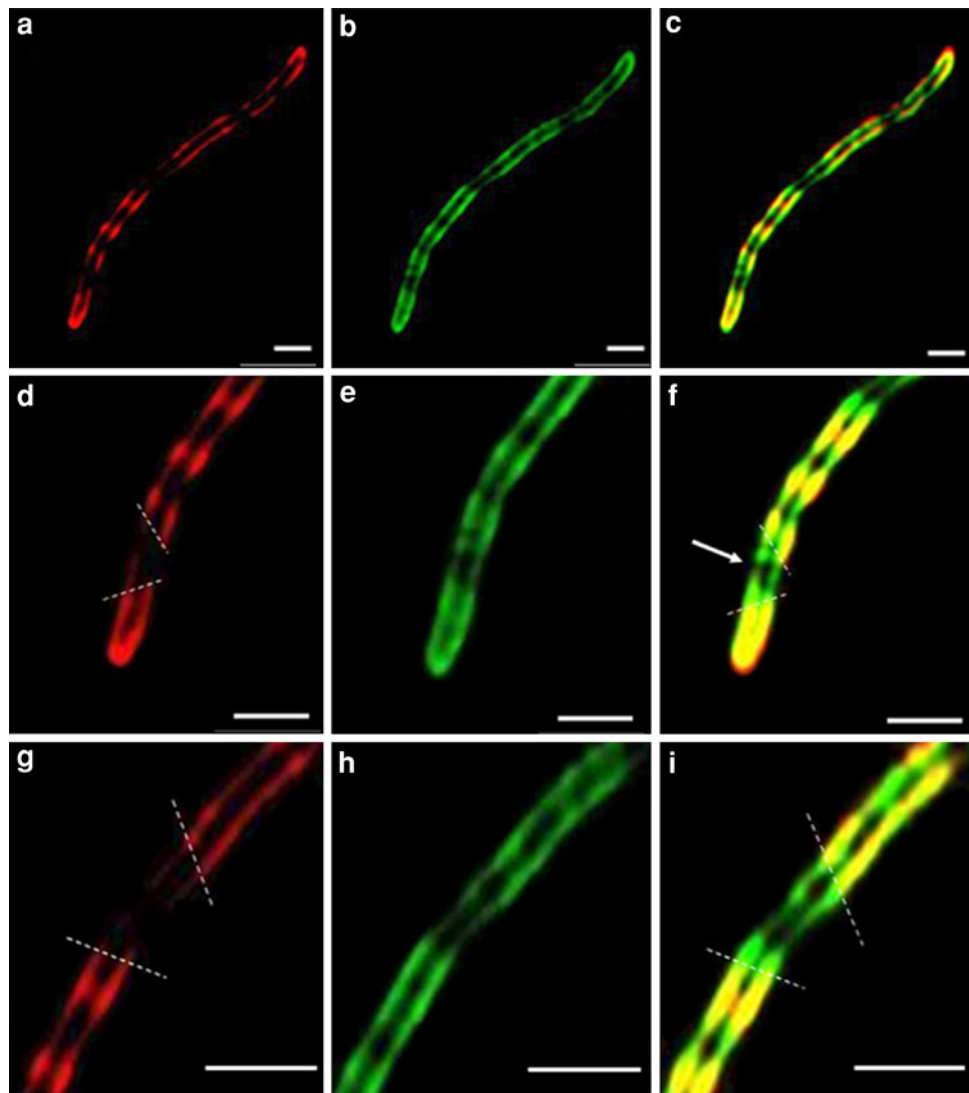
Fig. 4 Cell poles are inert for new OE synthesis. Cells from *T. thermophilus* were labelled with Texas Red and allowed to grow for 3 h at 70°C before being labelled again with Oregon Green to allow the detection of all cells. a Green channel (whole cells). b Red channel (old regions). Note that most Texas Red labelling is concentrated at cell poles

cell would be required. To check this point, we carried out a series of double labelling experiments, first with Texas Red and then with Oregon Green derivatives, on early exponential cultures of *T. thermophilus*. Cells labelled with Texas Red were allowed to grow for 30 min and subjected to labelling with Oregon Green, and then allowed to grow for an additional 30-min period. As shown in Fig. 5, the labelling with Oregon Green overlaps with that of Texas Red (see merged images in panel C) and extends enough towards the Texas Red unlabelled areas so as to define a central unlabelled region that results from the incorporation of new subunits to OE. The observed pattern supports the idea of a unique centrally located site of insertion for the new S-layer subunits. Insertion at the old-to-new edges should have generated two dye-free areas separated by a green-labelled central area, which was not observed.

Pattern of SlpA insertion

As shown in Fig. 3, the transitions from new (unlabelled) to old (labelled) regions of the cell envelope forms an angle of around 60° in several of the images analysed. This suggests that incorporation of the new subunits of SlpA follows a helical pattern. To confirm this, different optical sections of cells grown for one generation after labelling were deconvoluted and studied. As shown in Fig. 6, images formed top to bottom of dividing cells revealed that the polar labelled regions were not perpendicular to the axis, but formed a bevelled section with an angle of 60° . A 3D reconstruction movie of these images is shown in supplementary material. Another example of the presence of these bevelled growth observed in a bacteria seem to be in a 90° rotation (frontal view) with respect to the cell of Fig. 6 (lateral view) as shown in supplementary Fig. 1. In this case, the angle of SlpA growth area is

Fig. 5 New cell envelope takes place at central growing zone. Exponential cultures were labelled first with Texas Red, and after 30 min of growth, with Oregon Green, before being analysed by confocal microscopy. **a, d, g** Images to detect the first labelling (red channel) at increasing sizes. **b, e, h** Same images in the green channel. **c, f, i** Merge of the images of the two channels. The *white arrow* in **f** indicates the central growth region. *Dotted lines* in **d, g** and **i** show the apparent angle of the transition zone



detected by the changes in the length of the labelled regions observed in the optical sections. All these data strongly support a helical pattern of growth of the S-layer and, likely, the whole outer membrane of *T. thermophilus*.

Discussion

Due to the strong lateral interactions that form the symmetric structures of the S-layer, growth based on insertion of the new S-proteins should be accomplished in such a way as to minimize lateral rearrangements of the crystalline layer. Actually, the few analysis carried out so far point to two main models for the incorporation of S-proteins, either localized at the septal regions of the cells as in most Gram-positive bacilli so far analysed, or a diffuse one in which the new subunits are incorporated all over the cell surface as in the cell bodies of *Caulobacter crescentus*.

These different models are likely the consequence of structural constraints produced along the evolutionary story of the adaptation of the S-layer to the hosts. In Gram positives, the S-layer binds to a thick, relatively inert cell wall, so it is likely that its synthesis should concentrate at the regions of cell wall growth, whereas in Gram negatives the S-layer binds to a theoretically more dynamic structure, such as the LPS layer of the outer membrane.

Both the cell envelope and the properties of S-layer from *T. thermophilus* are different from those characteristics for Gram positives and Gram negatives. The Gram positive-like composition of the peptidoglycan and the presence of secondary cell wall polysaccharides contrast with the thinness of the sacculus and the existence of an outer membrane-like structure in which the SlpA protein acts both as a cell wall anchoring element and scaffold component. This dual role shows up in the SlpA amino acid sequence, which has properties common to Gram-negative



Fig. 6 Helical growth of the outer envelope. The cells were labelled with Oregon Green and allowed to grow for one generation before being subjected to confocal microscopy. Deconvoluted images from proximal (upper left) to distal (bottom right) optical sections of a dividing cell are shown. A 3D reconstruction of these images is shown as supplementary material

porins and Gram-positive S-Layers. Due to the phylogenetic position of this bacterium as a likely intermediate between Gram positives and Proteobacteria (Gupta 2000), the study of the mechanism of insertion of SlpA could give relevant information regarding how synthesis of the OM has evolved in the latter.

Due to the thermophilic character of this bacterium, and to the presence of an external polysaccharide-rich layer of material that mask SlpA (Castón et al. 1988), it was not possible to use antibodies to label tag-modified SlpA subunits expressed from plasmids (not shown). The chemical method used here was based on the formation of covalent bonds between amino groups and a fluorescent dye. The labelling conditions used were similar to those described to reveal the formation of multicellular bodies in mutants defective in *slpA* regulation (Castan et al. 2002) and were mild enough as to have no apparent effect on the viability of the cells. Actually, images taken by confocal microscopy confirmed that most fluorescence accumulated on the cell envelope with a lower amount concentrated on soluble proteins. It is likely that the size (around 817 Da) and negatively charged character of the fluorophores used decreased their permeability through membranes, at the relatively high pH used, so some of the labelled soluble proteins could correspond to periplasmic components. As SlpA is so abundant and lysine-rich (4% of its amino acids content), most of the fluorescence is concentrated on this protein or on its degradation products, as revealed in Fig. 1, by their reaction with monoclonal antibodies that do not recognize any protein in a $\Delta slpA$ mutant. The SlpA degradation products shown in Western blots are generated by

native thermostable proteases during the denaturation step used for the analysis of the samples by PAGE (Caston et al. 1996; Fernandez-Herrero et al. 1995). On the other hand, the low labelling of proteins in the $\Delta slpA$ mutant are likely the consequence of the formation of an outer envelope of different protein composition (rich in the SlpM protein) and separated from the cell wall forming what it is known as multicellular bodies (Olabarría et al. 1996).

In our *in vivo* labelling experiments, other cell envelope proteins are faintly labelled, but their signals are so low that only an overexposure allows their detection in the TX100 soluble fractions, a treatment that solubilizes most IM and several OM components (Caston et al. 1993). Therefore, our data demonstrate that most labelling is concentrated on the SlpA protein, and that the images of cells grown after the treatment define the pattern of incorporation of this protein to the OE of *T. thermophilus*.

The analysis of labelled samples after short growth times showed the incorporation of the new material as black regions at the centre of the cells, without any detectable gradient of fluorescence with respect to the cell poles that remain basically as labelled at the start of the experiment even after a few duplication events. This “fully conservative” model supports that the external envelope of *T. thermophilus* is basically inert to new synthesis or recycling processes once it has been synthesized, a view that fits with the conservation of 70% of the fluorescent label associated with the cells after 24 h of incubation. Therefore, the model seems more similar to what has been described for the few Gram positives, in which the incorporation of S-layer has been studied, than to the diffuse model proposed for *Caulobacter*. However, the model in *Thermus* seems more conservative than in other Gram positives, which release detached S-protein to the medium (Sara and Sleytr 2000) at levels that can be exceptionally high as in *Bacillus brevis* (Tsukagoshi et al. 1984). The insoluble nature of SlpA and the tight transcriptional and translational control exerted on its synthesis (Fernandez-Herrero et al. 1997) support that detachment of SlpA from the cell wall is a rare event that takes place only when the synthesis of SlpA is de-regulated (Castan et al. 2002) or when synthesis of the secondary cell wall polymer to which it binds is inhibited (Cava et al. 2004).

An intriguing fact of the analysis of growing cells is that the transition line between old and new (labelled–unlabelled) is not perpendicular to the longitudinal axis of the cells, but is instead tilted by roughly 60° with respect to the longitudinal axis of the cell (Figs. 3, 4, 5). A more in-depth analysis of cell sections observed from lateral (Fig. 6, supplementary movie) and frontal (Supplemental Fig. 1) views confirmed that the incorporation of new material forms an angle with respect to the axis of the cells. Such a tilted mode of synthesis suggests that incorporation of the

external envelope follows a helical pattern that could fit with the formation of a hexagonal array by SlpA. Such helical patterns have been observed for MreB actin-like filaments in different rod-shaped bacteria (Graumann 2007; Thanbichler and Shapiro 2008). These bacterial cytoskeleton components have been proposed to be involved in the correct location of the complexes involved in the “lateral” synthesis of peptidoglycan (Garner et al. 2011). However, whereas the synthesis of peptidoglycan is likely to occur at different positions within the bacterial cylinder (Harold 2007), and hence in a partially diffuse way, the synthesis of the outer membrane seems to concentrate at a central point within the cell, as revealed by the two-step labelling experiments shown in Fig. 5.

At a defined moment in the cell division cycle, the central region of the bacteria, where incorporation of OE takes place, differentiates to form the septum and the resulting new cell poles of the daughter cells. In such a period, it is likely that the septum-forming machinery becomes coordinated with that implicated in the synthesis of the OE–SlpA envelope. In addition, and likely depending on the phase and the rate of growth, new OE incorporation points have to be activated at the centre of the daughter cells, as it has been observed also in double labelling experiments. It is important to note, that this model of conservative growth of the outer envelope implies that the material at the poles remains unchanged for at least several cell division cycles.

In conclusion, our experiments demonstrate that the synthesis of the SlpA and by extension of the outer envelope of *Thermus thermophilus* takes place at a central region of the cell through a model similar to that of the S-layer of Gram-positive Bacilli and not in a diffuse form like the S-protein of the Gram-negative *Caulobacter crescentus* (Smit and Agabian 1982) or the porins and lipopolysaccharides of Gammaproteobacteria (Bos et al. 2007).

Acknowledgments This work has been supported by grants BIO2007-60245 and BIO2010-18875 from the Spanish Ministry of Science. An institutional grant from Fundación Ramón Areces to CBMSO is acknowledged. An FPI fellowship to F. Acosta is also acknowledged.

References

- Beveridge TJ, Pouwels PH, Sara M, Kotiranta A, Lounatmaa K, Kari K, Kerosuo E, Haapasalo M, Egelseer EM, Schocher I, Sleytr UB, Morelli L, Callegari ML, Nomellini JF, Bingle WH, Smit J, Leibovitz E, Lemaire M, Miras I, Salamiou S, Beguin P, Ohayon H, Gounon P, Matuschek M, Koval SF (1997) Functions of S-layers. *FEMS Microbiol Rev* 20:99–149
- Bos MP, Robert V, Tommassen J (2007) Biogenesis of the gram-negative bacterial outer membrane. *Annu Rev Microbiol* 61:191–214
- Castan P, Zafra O, Moreno R, de Pedro MA, Valles C, Cava F, Caro E, Schwarz H, Berenguer J (2002) The periplasmic space in *Thermus thermophilus*: evidence from a regulation-defective S-layer mutant overexpressing an alkaline phosphatase. *Extremophiles* 6:225–232
- Caston JR, Berenguer J, de Pedro MA, Carrascosa JL (1993) S-layer protein from *Thermus thermophilus* HB8 assembles into porin-like structures. *Mol Microbiol* 9:65–75
- Caston JR, Olabarria G, Lasa I, Carrascosa JL, Berenguer J (1996) Differential domain accessibility to monoclonal antibodies in three different morphological assemblies built up by the S-layer protein of *Thermus thermophilus* HB8. *J Bacteriol* 178:3654–3657
- Castón J, Carrascosa J, de Pedro M, Berenguer J (1988) Identification of a crystalline layer on the cell envelope of the thermophilic eubacterium *Thermus thermophilus*. *FEMS Lett* 51:225–230
- Cava F, de Pedro MA, Schwarz H, Henne A, Berenguer J (2004) Binding to pyruvylated compounds as an ancestral mechanism to anchor the outer envelope in primitive bacteria. *Mol Microbiol* 52:677–690
- Cava F, Hidalgo A, Berenguer J (2009) *Thermus thermophilus* as biological model. *Extremophiles* 13:213–231
- Engelhardt H, Peters J (1998) Structural research on surface layers: a focus on stability, surface layer homology domains, and surface layer–cell wall interactions. *J Struct Biol* 124:276–302
- Faraldo ML, de Pedro MA, Berenguer J (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
- Fernandez-Herrero LA, Olabarria G, Caston JR, Lasa I, Berenguer J (1995) Horizontal transference of S-layer genes within *Thermus thermophilus*. *J Bacteriol* 177:5460–5466
- Fernandez-Herrero LA, Olabarria G, Berenguer J (1997) Surface proteins and a novel transcription factor regulate the expression of the S-layer gene in *Thermus thermophilus* HB8. *Mol Microbiol* 24:61–72
- Garner EC, Bernard R, Wang W, Zhuang X, Rudner DZ, Mitchison T (2011) Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in *B. subtilis*. *Science* 333:222–225
- Graumann PL (2007) Cytoskeletal elements in bacteria. *Annu Rev Microbiol* 61:589–618
- Griffiths E, Gupta RS (2007) Identification of signature proteins that are distinctive of the *Deinococcus-Thermus* phylum. *Int Microbiol* 10:201–208
- Gruber K, Sleytr UB (1988) Localized insertion of new S-layer during growth of *Bacillus stearothermophilus* strains. *Arch Microbiol* 149:485–491
- Gupta RS (2000) The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiol Rev* 24:367–402
- Harold FM (2007) Bacterial morphogenesis: learning how cells make cells. *Curr Opin Microbiol* 10:591–595
- Howard LV, Dalton DD, McCoubrey WK Jr (1982) Expansion of the tetragonally arrayed cell wall protein layer during growth of *Bacillus sphaericus*. *J Bacteriol* 149:748–757
- Laemmli U, Favre M (1973) Maturation of the head of bacteriophage T4. I. DNA packaging events. *J Mol Biol* 80:575–599
- Messner P, Steiner K, Zarschler K, Schaffer C (2008) S-layer nanoglycobiology of bacteria. *Carbohydr Res* 343:1934–1951
- Mignot T, Mesnage S, Couture-Tosi E, Mock M, Fouet A (2002) Developmental switch of S-layer protein synthesis in *Bacillus anthracis*. *Mol Microbiol* 43:1615–1627
- Olabarria G, Fernández-Herrero LA, Carrascosa JL, Berenguer J (1996) *slpM*: a gene coding for an “S-layer like array” overexpressed in S-layer mutants of *Thermus thermophilus*. *J Bacteriol* 178:357–365

- Quintela JC, Pittenauer E, Allmaier G, Aran V, de Pedro MA (1995) Structure of peptidoglycan from *Thermus thermophilus* HB8. *J Bacteriol* 177:4947–4962
- Ramirez-Arcos S, Fernandez-Herrero LA, Berenguer J (1998) A thermophilic nitrate reductase is responsible for the strain specific anaerobic growth of *Thermus thermophilus* HB8. *Biochim Biophys Acta* 1396:215–227
- Sara M, Sleytr UB (2000) S-Layer proteins. *J Bacteriol* 182:859–868
- Sleytr UB, Glauert AM (1976) Ultrastructure of the cell walls of two closely related clostridia that possess different regular arrays of surface subunits. *J Bacteriol* 126:869–882
- Smit J, Agabian N (1982) Cell surface patterning and morphogenesis: biogenesis of a periodic surface array during *Caulobacter* development. *J Cell Biol* 95:41–49
- Thanbichler M, Shapiro L (2008) Getting organized—how bacterial cells move proteins and DNA. *Nat Rev Microbiol* 6:28–40
- Tsukagoshi N, Tabata R, Takemura T, Yamagata H, Udaka S (1984) Molecular cloning of a major cell wall protein gene from protein-producing *Bacillus brevis* 47 and its expression in *Escherichia coli* and *Bacillus subtilis*. *J Bacteriol* 158:1054–1060