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ORIGINAL PAPER

The β-barrel assembly machinery (BAM) is required for the assembly of a primitive S-layer protein in the ancient outer membrane of *Thermus thermophilus*

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Abstract The ancient bacterial lineage *Thermus* spp has a primitive form of outer membrane attached to the cell wall through SlpA, a protein that shows intermediate properties between S-layer proteins and outer membrane (OM) porins. In *E. coli* and related Proteobacteria, porins are secreted through the BAM (β-barrel assembly machinery) pathway, whose main component is BamA. A homologue to this protein is encoded in all the *Thermus* spp so far sequenced, so we wondered if this pathway could be responsible for SlpA secretion in this ancient bacterial model. To analyse this hypothesis, we attempted to get mutants on this BamAth of *T. thermophilus* HB27. Knockout and deletion mutants lacking the last 10 amino acids were not viable, whereas its depletion by means of a BamA antisense RNA lead defective attachment to the cell wall of its OM-like envelope. Such defects were related to defective folding of the SlpA protein that was more sensitive to proteases than in a wild-type strain. A similar phenotype was found in mutants lacking the terminal Phe of SlpA. Further protein–protein interaction assays confirmed the existence of specific binding between SlpA and BamAth. Taking together, these data suggest that SlpA is secreted through a BAM-like pathway in this ancestral bacterial lineage, supporting an ancient origin of this pathway before the evolution of the Proteobacteria.

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Introduction

The genus *Thermus* constitutes along *Deinococcus* one of oldest branches of the bacterial phylogeny either using 16S RNA (Hartmann and Erdmann 1989) or a combination of conserved proteins (Wu et al. 2009). The cell envelope of this bacterial group consists of a complex, multilayered structure. In *Thermus thermophilus*, a peptidoglycan sacculus of Gram-positive composition (Quintela et al. 1995) is surrounded by an outer membrane-like structure (OML) that generates a periplasmic space (Castán et al. 2002). Acting as scaffold for this OML, a hexagonal S-layer exists built up by the SlpA protein (Castón et al. 1988). Structural and biochemical evidence, as well as sequence analysis, indicate that SlpA represents a peculiar and, most likely, ancestral type of structural protein with properties shared by modern S-layers and OMPs (e.g., porins) from Proteobacteria (Engelhardt and Peters 1998). Actually, SlpA is water insoluble as OMPs, and associates in regular structures similar to those built up by bacterial porins under defined conditions instead of forming the hexagonal array exhibited in vivo as S-layer (Castón et al. 1993). As other OMPs from primitive bacteria (e.g., *Thermotogales*) SlpA contains a single S-layer homology domain (SLH) at its N-terminus instead of the three usually found in most S-proteins from Gram positives (Engelhardt and Peters 1998). It has been shown that the single SLH domain of SlpA is necessary and sufficient for the attachment of the OML to the cell wall (Olabarría et al. 1996), suggesting that it constitutes an ancestral form to attach a primitive OM to the cell.

Integral outer membrane β-barrel proteins (βOMPs) are among the most abundant proteins in Proteobacteria (Bos et al. 2007). In these bacteria, they play a wide variety of roles, such as channels for low molecular weight compounds, elements of complex machinery required for the secretion of proteins or toxic substances, enzymes, adhesion molecules, assembly factors, or structural ones like anchoring the OM to the cell wall. Despite such functional diversity proteobacterial βOMPs are synthesized, secreted through the cytoplasmic membrane and incorporated into the OM by following a common pathway. Their secretion through the inner membrane (IM) depends on SecB and requires an N-terminal signal sequence. As described for *E. coli*, it is generally assumed that once in the periplasm the nascent βOMPs peptide is bound by small periplasmic chaperones and enzymes (Eppens et al. 1997) like Skp, SurA, or DegP that avoid their aggregation and/or assist their folding (Bos et al. 2007; Knowles et al. 2009; Hagan et al. 2011). For the insertion of OMPs into the OM, the complex of the βOMP–chaperone is delivered to an OM complex known as BAM (from β-barrel assembly machinery). In *Neisseria meningitidis* it was determined for the first time that a βOMP protein of around 85 kDa (BamA, formerly Omp85) was essential for the viability of the bacteria (Voulhoux et al. 2003). The BamA protein had homologues in all Proteobacteria, and its coding gene was in many cases flanked by the *skp* gene and by a homologue of *rseP*, encoding a protease induced upon accumulation of unfolded βOMPs in the periplasm, a fact that is produced when BamA is depleted (Voulhoux et al. 2003). Similar properties were further described for the *E. coli* BamA homologue YaeT. In both bacteria, it has been shown that BamA is part of a multiprotein complex whose individual components have no well-defined functions, being only essential for *E. coli* the BamA and BamD proteins (Wu et al. 2005). Among other bacterial phyla, BamA homologues are found in all those phyla that contain an OM (*Deinococcus-Thermus*, *Cyanobacteria*, *Spirochetes*, *Chlorobi*, *Proteobacteria*, *Planctomycetes*, *Bacteroidetes*) except in *Chloroflexi*, being used by (Cavalier-Smith 2006) as marker to support transition analysis in phylogenetic studies of bacterial evolution. No homologues of BamA are present in *Firmicutes*, *Actinobacteria,* or in Archaea. However, BamA homologues are found in eukaryotes where they play similar functions in the insertion of OMPs with β-barrel structure into the OM of chloroplast and mitochondria (Bos et al. 2007).

The BamA homologue of *T. thermophilus* HB27 ($BamA^{th}$ thereafter for clarity) has been overexpressed and purified from *T. thermophilus* and shown to integrate as monomer into artificial lipid membranes, where it forms ring-like structures that are functional as ion channels (Nesper et al. 2008). As BamA this thermophilic counterpart contains five N-terminal POTRA domains and a C-terminal β-barrel domain that is predicted to contain 16 beta-sheets. Recently, it has been shown by pull-down experiments that $BamA^{th}$ interacts with TtoA, a small and abundant OML protein of *T. thermophilus* of unknown function whose structure has been solved (Brosig et al. 2009).

Having in mind the intermediate properties of the SlpA between bona fide S-layer proteins and porins we wondered whether $BamA^{th}$ could participate in its assembly. Our data support that $BamA^{th}$ is an essential protein whose depletion produces severe defects in the folding of the SlpA protein. In addition, we demonstrate direct protein–protein interactions between SlpA and $BamA^{th}$. These data support that the BAM pathway is ancestral and was likely generated during the evolution from primitive protective-only S-layers to the OML envelopes present in *Thermus* and, likely, in other ancestral bacterial lineages.

Materials and methods

Strains and growth conditions

Thermus thermophilus HB27 was grown at 60 or 70 °C in rich medium (TB) (Ramírez-Arcos et al. 1998) under strong aeration. The *E. coli* strain DH5α (*supE44 ΔlacU169 ϕ80 lacZΔM15 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was used for genetic constructions. *E. coli* was grown in LB at 37 °C. Plates containing agar (1.5 % w/v) were usually incubated in a water-saturated atmosphere for 24–72 h. Transformation of *T. thermophilus* was achieved by natural competence on growing cells (Koyama et al. 1986; de Grado et al. 1999), whereas standard protocols were used to transform *E. coli*. Selection was carried out on 1.5 % (wt/vol) agar plates with kanamycin (30 mg/L).

Isolation of mutants from *T. thermophilus*

Fragments of the *slpA* (code TTC1532) or *bamA* (code TTC0193) [previously published as Omp85(Tt) in Nesper et al. (2008)] genes coding for the C-terminal parts of the respective proteins were amplified by PCR with the appropriate primers (Table 1), and cloned into the suicide pK18 vector (Cava et al. 2004) to provide the target for homologous recombination. All the fragments were sequenced to confirm the absence of mutations others than those attempted. The vectors constructed were used to transform *T. thermophilus* HB27 by natural competence, and kanamycin-resistant colonies grown for 72 h were tested by PCR for the presence of the expected mutation in the gene with primers external to the inserted fragment (Table 1). When positive amplification was obtained the PCR fragment was sequenced to confirm the nature of the mutation obtained.

Table 1 Oligonucleotides used in this work

Name	Sequence 5′ > 3′	Purpose
Δomp85-dir-E	AAAAGAATTCTCCCCTGGCTCTACC	Forward for *bamA*
ΔFomp85-rev-X	TTTTTTCTAGATTACATGGGCCCGATGC	Reverse for ΔF
Δ10omp85-rev-X	TTTTTCTAGATTACCCCGTGGGGCT	Reverse for Δ10
ΔslpA-dir-E	AAAAGAATTCCTGAGCGGCCTGGAGATC	Direct for *slpA*
ΔFslpA-rev-X	TTTTTCTAGACTACTCCACGTTGTAG	Reverse for ΔF
Δ10slpA-rev-X	TTTTTCTAGACTACCGGCCGTAGGACTGGAC	Reverse for *slpA* Δ10
slpA-T	TTTTTCTAGACTACCACTCCACGTTGTAG	Reverse for F to W
Omp1400dir	CCTTCATCCAGAACGAC	Integration
SlpA1800dir	CCGACAACAAGGGCTTC	Integration
M13dir	CGCCAGGGTTTTCCCCGTCACGAC	Integration
asOmp85dirE	AAAAGAATTCTTTGGGTAAGCTTCCG	Direct for antisense *omp85*
asOmp85revX	AAAATCTAGAGGGGTGTAGACCTC	Reverse for antisense *omp85*

Antisense expression vector

Bifunctional *E. coli–Thermus* plasmid pMK184, a derivative of pMK18 (de Grado et al. 1998), was used to insert a fragment (1 kbp) of *bamA,* amplified from the *T. thermophilus* HB27 genome with the primers asOmp85dirE and asOmp85revX (Table 1), in reverse orientation respect to the kanamycin nucleotidyl transferase (*kat*) gene to allow constitutive transcription of its complementary strand (antisense) as described (Moreno et al. 2004).

Microscopy

Wild type and mutants of *T. thermophilus* HB27 were grown at 60 °C under mild stirring conditions. Samples were observed and recorded under phase-contrast optical microscopy.

Preparation of cell envelopes

400 ml of mid-log-phase cultures were harvested by centrifugation (5000×*g*, 10 min), cells were suspended to the initial volume in 10 mM Tris–HCl (pH 7.8) buffer, centrifuged as above, and resuspended in the same buffer containing 1 mM EDTA. Cells were broken by French Press (12000 Ib/In^2, two cycles). Unbroken cells were removed by low-speed centrifugation (5000×*g*, 30 min), and cell envelope fragments were recovered by high-speed centrifugation (30000*g*, 30 min). The insoluble fraction was washed three times by centrifugation in the same buffer (30000*g*, 30 min) before being resuspended in 2 ml of the same buffer at a concentration of around 10 μg/μl. All manipulations were performed at 4 °C. Cell envelope aliquots were used immediately or kept at −70 °C until needed.

Solubilization and immunodetection of cell envelope proteins

Cell envelope fractions were incubated for 15 min at 60 °C in five volumes of 0.5 % (vol/vol) Triton X100 (TX100), and soluble and insoluble fractions were separated by centrifugation (30000*g*, 30 min) with two additional washing steps under the same conditions. TX100 soluble and insoluble fractions were boiled in Laemli's denaturing buffer (100 °C × 10 min), and separated by SDS-PAGE (Laemmli and Favre 1973) before being stained with Coomassie brilliant blue or subjected to immunodetection by Western blot. For this, proteins separated by SDS-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane and subjected to incubation with a polyclonal rabbit antiserum against the S-layer protein from *T. thermophilus* NAR1 (Faraldo et al. 1991) or against the BamAth protein (Nesper et al. 2008). After washing, the presence of the antibodies bound to the membrane was detected by bioluminescence with mouse monoclonal anti-rabbit antibodies coupled to horseradish peroxidase (ECL, Amersham International).

Protease sensitivity assays

Purified membrane fractions from the analysed strains containing 1.5 μg of proteins in 40 μl were subjected to 3-min incubation with Proteinase K from *Tritirachium album* (111 ng of protein corresponding to 4.87 × 10^{-3} U) in the presence of different concentrations of 2 and 4 M urea in 25 mM Tris–HCl, 5 mM $CaCl_2$, pH 7.5. The reactions were stopped by boiling in Laemli sample buffer and the proteins separated by SDS-PAGE and identified by Western blot. Monoclonal antibody 1AE1 and a rabbit polyclonal antiserum were used to detect SlpA and BamAth, respectively.

Far Western blotting assays

For Far Western affinity assays cell envelope preparations containing 10 μg of proteins (including the prey protein) were heated at 90 °C for 1 min in Laemli's sample buffer, loaded onto SDS-PAGE gels and subjected to separation under mild electric field (10–25 V, 3–5 mA) for 5 h at 4 °C. Separated proteins were transferred to a nitrocellulose (0.2 μm, Amersham) filter in transfer buffer (50 mM Tris, 192 mM glycine, 20 % v/v methanol) for 6 h at 4 °C (10 V, 25 mA). The filter was then incubated in renaturation buffer (20 mM Tris–HCl, 150 mM NaCl, 2,5 mM DTT, 2,5 % v/v NP-40, 10 % v/v glycerol, 5 % p/v BSA) for 16 h at 4 °C. In the binding step, filters were incubated with the TX100-soluble extracts from membrane aliquots of the *ΔslpA* mutant containing 5 mg of proteins. Incubation was carried out in binding buffer (10 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 % v/v glycerol, 0.125 % p/v BSA) containing 0.2 % of SDS. Finally, the presence of the bait $BamA^{th}$ protein bound to specific prey proteins was detected with antiserum against $BamA^{th}$ (Nesper et al. 2008).

Results

Depletion of $BamA^{th}$ affects coordination in the synthesis of the cell envelope

To test the putative role of $BamA^{th}$ in the expression of SlpA we attempted to knockout the corresponding gene. For this, we followed a protocol of insertional mutagenesis by double recombination with a gene conferring thermostable resistance to kanamycin similar to that described to inactivate several other genes of *T. thermophilus* in previous works (Lasa et al. 1992; Fernandez-Herrero et al. 1995; Ramírez-Arcos et al. 1998). However, all the attempts were unsuccessful, as no colonies grew on kanamycin plates upon several transformation experiments when lineal DNA constructs carrying the selectable deletion was used. Thus, an alternative strategy of single recombination was carried out by using derivatives of suicide vector pK18 containing internal fragments of the *bamA* gene. With this method, a small number of kanamycin-resistant colonies grew (around 20 colonies/μg). However, PCR analysis revealed the presence of an entire wild-type *bamA* in addition to that of the *kat* gene and plasmid regions, as expected from the integration of the plasmid (not shown). Similar results were repeatedly obtained in all our transformation assays supporting that integration of the constructs took place at different points in the genome. These data lead us to conclude that $BamA^{th}$ was an essential protein in *T. thermophilus*.

With this in mind, we attempted to decrease the expression of the *bamA* gene using an antisense RNA strategy already used with the catalase gene (Moreno et al. 2004). For this, we transformed the wild-type strain with a construct expressing constitutively a *bamA* antisense RNA from plasmid pMK184-bamA ("Materials and methods"). This time transformation rendered a significant number of kanamycin-resistant transformants (10^4 colonies/μg) after 3 days of incubation, supporting that the plasmid was not lethal for the cells. Western blots from fresh cultures of clones expressing the antisense *bamA* RNA revealed that the amount of $BamA^{th}$ expressed by these cells was lesser (<50 %) than that of control cells transformed with the empty vector (Fig. 1). Clones expressing the *bamA* antisense RNA showed slow growth and the formation of multicellular bodies (MBs) in liquid medium (Fig. 2b). Such MBs have been described previously in different *T. thermophilus* mutants. Either overexpression (Castán et al. 2002) or absence (Lasa et al. 1992) of SlpA leads to the defective attachment of the OML to the cell wall, especially at septation sites, leading to the formation of groups of cells surrounded by a common OML through further cell divisions without segregation. Undistinguishable phenotypes are also observed in mutants lacking the SLH domain of SlpA (Olabarría et al. 1996), and in mutants lacking a cell wall pyruvilating enzyme required for the binding of SlpA (Cava et al. 2004). Therefore, the formation of MBs observed suggested that $BamA^{th}$ depletion was affecting SlpA expression and/or folding, leading to OML detachment.

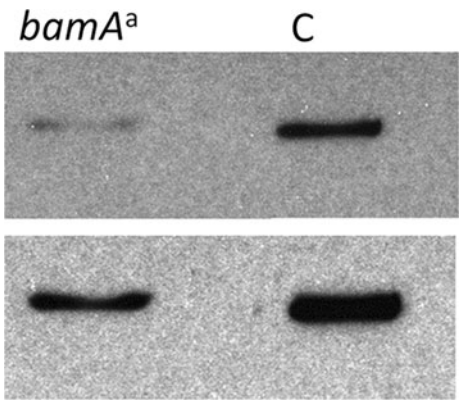

Fig. 1 Depletion of $BamA^{th}$ expression by antisense RNA. Western blot to detect $BamA^{th}$ in membranes (2 μg of proteins) from cultures of *T. thermophilus* cells harbouring plasmid pMK184 (*C*) or a derivative expressing an antisense *bamA* RNA ($bamA^a$). Two exposures are shown to better compare the differences in protein signal

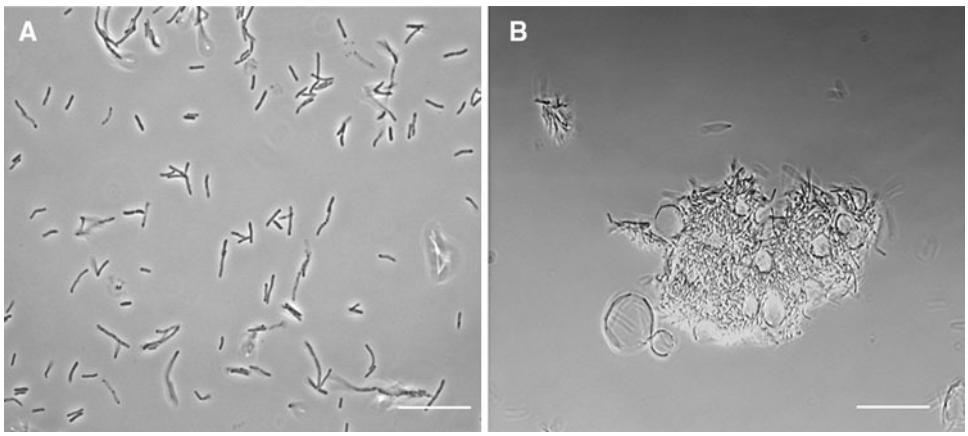

Fig. 2 Effects of *bamA* antisense expression. **a** Wild-type strain transformed with pMK184. **b** Cells expressing antisense *bamA* RNA. *Bars* 10 μm

BamAth is required for proper SlpA folding

To test the hypothesis above, we purified membranes from exponential cultures of *T. thermophilus* carrying either the plasmid expressing the antisense *bamA* RNA or its empty control, and subjected identical amounts of membrane protein from each sample to digestion with Proteinase K.

As shown in Fig. 3, in the presence of 2 M urea, Proteinase K produced degradation of SlpA in membranes of both cultures (lanes 2 and 6), although the protein was slightly more resistant in cells transformed with the control plasmid. At 4 M urea, the protease hydrolysed SlpA from cells expressing the antisense (lane 4), but not that of control cells (lane 8), thus supporting differences in SlpA folding between both cultures.

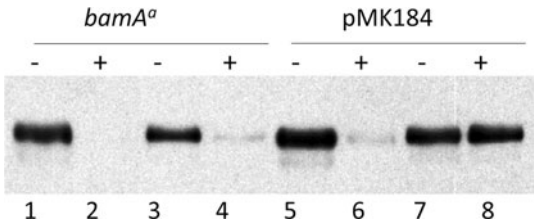

Fig. 3 Sensitivity of SlpA to Proteinase K. Western blot to detect SlpA with monoclonal antibody 1AE1 on whole membrane aliquots (0.15 μg of proteins at final concentration of 30 μg/ml) from *T. thermophilus* exponential cultures transformed with pMK184 or with a derivative expressing the antisense *bamA* RNA (*bamA*^{a}). Assays were carried out in presence of 2 (*1*, *2*, *5*, *6*) or 4 M urea (*3*, *4*, *7*, *8*), and samples were incubated for 3 min with (+) or without (−) Proteinase K (17 ng, final concentration of 3.4 μg/ml)

Interaction between SlpA and BamAth

The results above support that proper SlpA folding depends on BamAth. If this was true, an interaction between both proteins should occur. As SlpA forms multimeric regular structures insoluble in neutral detergents (Castón et al. 1993), samples containing the prey SlpA protein were transferred to nitrocellulose membranes, subjected to a re-folding treatment, and incubated with samples containing the detergent-solubilised bait protein (BamAth). The presence of such bait protein bound to the membrane was detected with a specific anti-BamAth antiserum. We used solubilised envelopes of a *ΔslpA* mutant as SlpA-free source of the BamAth bait protein (Fernandez-Herrero et al. 1995). As source of the prey SlpA protein, we used Triton X-100 (TX100)-insoluble fractions from membranes of the wild-type strain because BamAth is soluble in this detergent and can be extracted within the soluble fraction.

As shown in Fig. 4a the SlpA protein was identified by Western blot with anti-SlpA polyclonal antiserum in the TX100-insoluble fraction of cell membranes from the wild-type strain subjected to one (lane 3) or two (lane 2) treatments with TX100, whereas it was absent from membranes of the *ΔslpA* mutant (lane 1). Smaller protein bands detected by the polyclonal antiserum against SlpA correspond to proteolytic products of the protein, as demonstrated by their absence in the *ΔslpA* mutants and by their detection with monoclonal antibodies (Olabarría et al. 1996). A parallel Western blot revealed the presence of BamAth (Fig. 4b) in the membranes of the *ΔslpA* mutant (lane 1), and only traces of this protein in the insoluble fraction of the wild-type strain after a single extraction with the detergent (lane 3). These traces were not detected after a second extraction with TX100 (lane 2). When the filters were incubated with membranes of the *ΔslpA* mutant solubilised with TX100 (our source of BamAth without any trace of SlpA), we detected the presence of BamAth (Fig. 4c) on a protein band with electrophoretical mobility corresponding to the position of SlpA (lanes 2 and 3), thus showing that BamAth interacts with SlpA. The protein band of slightly lesser size in lane 1 corresponds to BamAth in the *ΔslpA* mutant (compare panels b and c). Binding of BamAth to two additional protein bands of much smaller size (around 70 and 40 kDa) was also detected (line 3, white arrowheads). The larger of these two proteins is

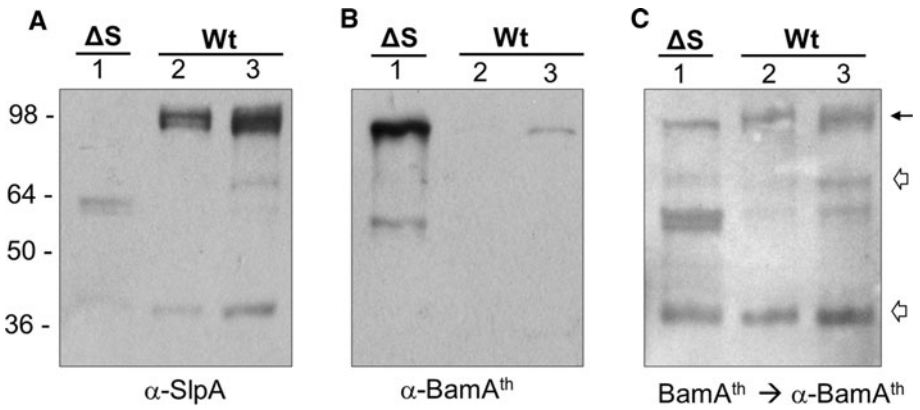

Fig. 4 Interaction between SlpA and BamAth. Total membrane proteins from the *ΔslpA* mutant (*1*) and proteins from the insoluble fraction of membranes from the wild-type strain extracted one (*3*) or two times (*2*) with TX100 were separated by SDS-PAGE, transferred to nitrocellulose filters and immunoassayed for the presence of SlpA (**a**) or BamAth (**b**). In a parallel experiment the filter was incubated with solubilized membranes of the Δ*slpA* mutant and further subjected to immunodetection of BamAth (**c**). *White arrowheads* point to protein bands of approx. 70 and 40 kDa to which BamAth binds in addition to SlpA (*black arrowhead*). Protein size markers (kDa) are labelled on the *left panel*

absent from the *ΔslpA* mutant, but detected with α-SlpA (Fig. 4a, lane 3), supporting that it might correspond to a degradation product of SlpA, whereas the smallest one, detected also in the mutant, corresponds to an unknown TX100-insoluble protein recognized by BamAth, likely a beta-barrel OM protein. In conclusion, these assays demonstrate the existence of specific interactions between SlpA and BamAth.

S-layer proteins from members of the *Deinococcus–Thermus* phylum contain C-terminal signatures like those of *β*-barrel OM proteins from Proteobacteria

The data above support that the BAM pathway for integration of OM *β*-barrel protein in modern Proteobacteria was responsible for S-layer integration into the ancestral envelope of *Thermus thermophilus*. To evaluate whether this was a general property of the ancient phylum to which this species belongs, we searched for SlpA homologues among the sequenced strains of members of the *Deinococcus–Thermus* phylum. This analysis revealed the presence of SlpA homologues in different *Thermus* spp strains, and in *Deinococcus* spp, *Marinithermus hydrothermalis*, *Oceanithermus profundus* and *Meiothermus silvanus.* In these other genus the similarity to SlpA was essentially limited to the S-layer homology domain (SLH) located at their respective N-terminus (positions 1–190) (Engelhardt and Peters 1998). In addition to this common domain, SlpA homologues showed sequence similarities over the last amino acids of their C-terminus, in which a signature sequence was found identical to that described as required for the correct synthesis of *β*-barrel OMPs secreted through the BAM pathway in Proteobacteria (hXhXhXhXF, where h means any hydrophobic amino acid) (Fig. 5). Such

SlpA Tth HB27	QSYGRAFKVTYNVEF$_{948}$
SlpA Tth HB8	PNFGRGFKISYTVKF$_{928}$
SlpA Tth NAR1	QTHGQAFKISYTVKF$_{917}$
SlpA Tth PRQ25	PEHGRGFKINYTVKWGSRSTTP$_{915}$
SlpA Tth SG	RSYGRAFKVTYNVEF$_{942}$
SlpA-l Tsco	GTYGRVFRINYTVNF$_{887}$
SLH-P Taq	GDHARTFRVTYTVKF$_{910}$
SlpA Tth JL-18	GDHARTFRVTYTVKF$_{894}$
SLH-P Msil	DSVGRGFRIFYNVSF$_{919}$
S-layer Drad	PARGQTFKISYKVNF$_{1167}$
SLH-P Dmar	AATGQSFKISYKVNF$_{1097}$
SLH-P Des	QSVAQGFKVGYSFRF$_{880}$
SLH-P Opro	TTHGGAFKVSYSVEF$_{891}$
SLH-P Mahy	CTHAQAFKMAYEVEFGEK$_{916}$
Omp85 Tth HB27	ESPTGRIHFRIGPMF$_{822}$
Consensus	**hXhXhXhXF**

Fig. 5 Alignments of the C-terminal sequence of actual and putative S-layers of the *Deinococcus–Thermus* phylum. The C-terminal sequences of SlpA and homologues from *T. thermophilus* strains HB8 (TTHA1893), HB27 (TTC1532), NAR1 (X57333), PRQ25 (CCE60595.1), SGO.5JP17-16 (AEG34288) and JL18 (TtJL18_1978), *T. aquaticus* Y51MC23 (EED10139), *T. scotoductus* SA1 (TSC c24210), and putative S-layer proteins from *Deinococcus radiodurans* R1 (DR2577), *D. deserti* VCD115 (Deide20610), *D. maricopensis* DSM21211 (Deima 0555), *Marinithermus hydrothermalis* DSM14884 (Marky 0134), *Oceanithermus profundus* DSM14977 (Ocepr 0326) and *Meiothermus silvanus* DSM 9946 (Mesil 0107) were compared. The BamAth (TTC0193, Omp85) from *T. thermophilus* HB27 has been included in the alignment along the consensus sequence for proteins transported through the BAM pathway in Proteobacteria

sequence was also found in BamAth. The exceptions to this signature were the SlpA protein homologues from *T. thermophilus* PRQ25 and *Marinithermus hydrothermalis*

DSM14884, which show 7 and 3 amino acid extensions, respectively and the replacement of F by W in the first.

Requirement for the C-terminal sequences for SlpA synthesis

In an attempt to analyse the relevance of this C-terminal conserved signature in the SlpA protein of the genetically amenable *T. thermophilus* HB27 strain, we constructed in vitro deletion forms lacking 1, 10, and 100 amino acids form its C-terminus and used them as recombination arm within suicide plasmids (pK18 derivatives). Only constructs lacking the last Phe were successfully integrated into the target *slpA* gene, whereas deletions of 10 or more amino acids were not viable. A phenotypic analysis of the *slpAΔF* mutant revealed the presence of MB similar to those produced by the expression of antisense *bamA* RNA (Fig. 6a), and concomitant differences in the pattern of SlpA protein degradation compared to the wild-type strain (Fig. 6b). We concluded that this SlpAΔF mutant protein has folding problems that make it not completely functional in vivo. Even a single replacement of Phe by Trp was not viable, suggesting that whereas absence of terminal Phe in SlpA was tolerated, its replacement by a bulkier amino acid generates a toxic product.

Discussion

The cell envelope of members of the *Deinococcus–Thermus* phylum is dramatically different from the structural and biochemical point of view from Gram-positive (Firmicutes) and Gram-negative (Proteobacteria) models. The presence of an OML in *Thermus* spp as detected by electron microscopy (Castón et al. 1988) was contradictory to the identification of a peptidoglycan composition more like that of Gram positives (Quintela et al. 1995) and with the presence of a thick "intermediate" layer of "secondary" polysaccharides covalently bound to it. The attachment of this OML to the intermediate layer was also unusual, as it was mainly dependent on the binding of the SlpA through its SLH domain, a fact also reminiscent of the binding of exoenzymes to the cell wall of Firmicutes (Engelhardt and Peters 1998). SlpA is one of the most abundant proteins in the cell envelope of *T. thermophilus*. It forms a regular (hexagonal) structure that covers the cell and shows structural (Castón et al. 1993) and sequence (Engelhardt and Peters 1998) properties common to both S-layers and outer membrane porins from Proteobacteria, acting as a sort of scaffold structure for the entire OML.

Due to the ancient phylogenetic position of *Thermus* spp (Wu et al. 2009; Cavalier-Smith 2006) it is likely that its OML and the mechanism for its synthesis are ancestral to that found for the OM from modern Proteobacteria. In these, the synthesis of β-barrel OMPs depends on the BAM protein complex that allows their proper insertion through a yet not completely understood mechanism (Bos et al. 2007), in which the BamA protein is absolutely required. In this article we show that secretion of SlpA depends on a protein of the BamA family encoded by *T. thermophilus* (Nesper et al. 2008).

The first clue on the biological relevance of this BamAth protein for *T. thermophilus* was derived from the unsuccessful attempts to get mutants by single recombination, supporting that as its *E. coli* counterpart (Doerrler and Raetz 2005; Werner and Misra 2005) BamAth is essential for the cell. Since the high recombination frequency shown by this thermophile and the limitations in the availability of controlled promoters precluded a direct demonstration of such essentiality similar to that used with *E. coli*, we

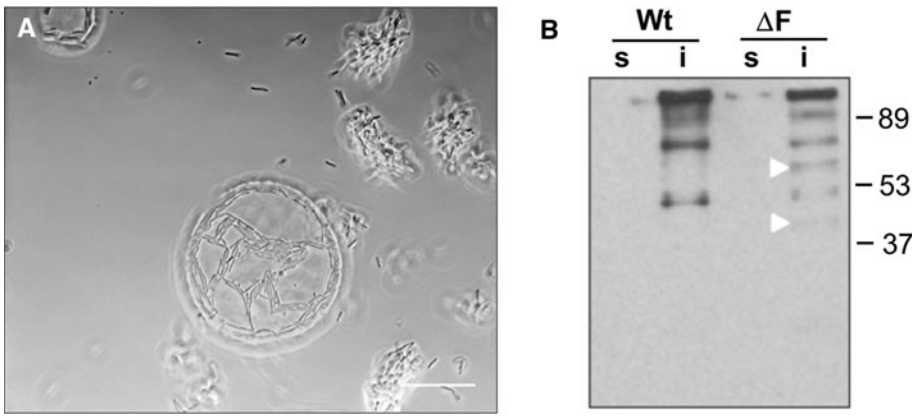

Fig. 6 The *slpAΔF* mutant shows similar defects as cells expressing antisense *bamA* RNA. **a** Phase-contrast images of exponential cultures of the *slpAΔF* mutant of *T. thermophilus* HB27. *Bar* 10 μm. **b** Western blot with polyclonal rabbit antiserum to detect SlpA and its products in TX100-soluble (*s*) and insoluble (*i*) fractions of membranes from the wild type (*Wt*) and its *slpAΔF* mutant. *Numbers on the right* indicate the mobility of protein size markers

decided to use the expression of an antisense RNA to decrease the amount of BamAth.

This strategy was shown to decrease the amount per cell of the targeted protein (Moreno et al. 2004) and constitutes an excellent method to analyse the role of essential proteins in *T. thermophilus*. In our hands, expression of *bamA* antisense renders cells with less than half the amount of BamAth per cell with respect to the wild-type strain. However, it is relevant to note that for this strategy to work the analysis of the transformed cells has to be carried out on freshly transformed cells, as further growth selects for derivatives that compensate the amount of BamAth synthesized by an unknown mechanism, leading to the recovery of a wild-type cell morphology (not shown).

In these BamAth-depleted cells, the OML does not bind properly to the underlying cell wall and the cells keep dividing inside sharing the external envelope, a kind of phenotype very similar to that produced by different mutations in SlpA. Actually, the SlpA protein from BamAth-depleted cells was more sensitive to proteases than that from the wild type, supporting that SlpA folding was not identical in both strains. In these assays (Fig. 3) differences in sensitivity were especially relevant in 4 M urea, being the SlpA in the wild-type strain more resistant than in the mutant, and thus supporting a stronger folding in the wild type. Why these differences in sensitivity were not so well detected at lower urea concentrations are not clear, but are likely related to a decrease in the activity of the protease itself.

In addition to the differences in SlpA sensitivity to proteases, different binding assays with membrane fractions showed the existence of specific binding SlpA-BamAth interactions, but the similar size between both proteins made difficult the interpretation of the preliminary results. Therefore, in experiments like that shown in Fig. 4 we used TX100-soluble and insoluble membrane fractions to separate both proteins before the binding assays and also a mutant lacking the SlpA protein as a "clean" source of BamAth. Our results showed that BamAth binds to membrane-immobilized SlpA in a specific manner. In these experiments other protein bands were also detected in the *ΔslpA* mutant that could correspond to other proteins that require BamAth for secretion. The only protein that has been shown previously to bind BamAth is TtoA (TTC0834), an OML protein from *T. thermophilus* HB27 with an electrophoretical mobility around 23 kDa that has been crystallized (Brosig et al. 2009). In our assays, we did not detect any 23 kDa protein. Instead, another TX100-insoluble protein of around 50 kDa was detected, which nature does not correspond to a SlpA fragment because it was also detected in the *ΔslpA* mutant (Panel A, lanes 2 and 3). A search of the genome of *T. thermophilus* HB27did not render any clue on the nature of such a 50-kDa protein.

In the work by Brosig et al. (2009), efficient binding of TtoA to BamAth depended on the presence in the protein of a C-terminal 9-amino-acid sequence that follows the same consensus pattern described for secretion of *β*OMPs in Proteobacteria. A similar pattern is also present at the C-terminus of SlpA and in many other putative S-layer-like proteins of the *Deinococcus–Thermus* phylum that share with SlpA a SLH domain at their respective N-terminus. Having in mind that SlpA is not essential for cell survival (Lasa et al. 1992), our inability to get SlpA mutants without the 10 last amino acids supports that expression of SlpA proteins lacking this C-terminal motif is toxic to the cell, likely because of the accumulation of improperly folded intermediates. Even the deletion of the last Phe produced cell defects similar to those produced by the depletion of BamAth (Fig. 6) supporting that this residue contributes to achieving an appropriate interaction with BamAth but is not essential for it. In contrast to this tolerance of F-terminal deletions, replacement of this F by W was toxic for the cells despite the fact that this amino acid is present in the signature sequence of the SlpA-like proteins of *T. thermophilus* PRQ25 (Fig. 5). It is likely that in the case of this strain the 7-amino-acid C-terminal extension compensates for a putative decrease in affinity for BamAth produced by the Phe to Trp change. In this sense, it is also relevant to note that in Proteobacteria there are examples of proteins, like OmpA of *E. coli*, in which this signature amphipathic *β*-strand is found in an internal region of the protein (Klose et al. 1988).

In addition to its interaction with BamAth, interactions with other unknown proteins are likely relevant for proper incorporation of SlpA to the OML. In this sense, it has been recently shown that SlpA synthesis localizes to central regions of the cells following a helicoidal pattern and not in a diffuse manner (Acosta et al. 2012). This means that secreted SlpA has to be kept partially unfolded and protected from proteases to diffuse to its final integration place in the OML before complete folding of its SLH domain could lead to its high-affinity anchoring, and concomitant immobilization to the underlying cell wall. Nothing it is known about such putative chaperones in *T. thermophilus*, and no homologues to chaperones of Proteobacteria implicated in the secretion of *β*OMPs like SurA, DegP, or Skp are encoded in its genome. In any case, given that once integrated into the OML the strength of horizontal self-interaction within SlpA subunits blocks any possibility of lateral diffusion, we had to hypothesize that BamAth has also to be located at these central OML growth sites.

In conclusion, our data support that a mechanism for *β*OMPs protein secretion similar to that already known in modern Proteobacteria was evolved in ancestral microorganisms like the *Deinococcus–Thermus* phylum for the secretion of the S-layer scaffold of its primitive form of

outer membrane. It is likely that the complexity of this ancient BAM-dependent secretion pathway in terms of proteins involved should be lesser than in modern Gram negatives. Available technologies for *T. thermophilus* HB27 (Cava et al. 2004) will facilitate its genetic and biochemical analysis.

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