Extremophiles (2012) 16:485–495
DOI 10.1007/s00792-012-0448-x

ORIGINAL PAPER

Homogeneous incorporation of secondary cell wall polysaccharides to the cell wall of *Thermus thermophilus* HB27

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Received: 6 February 2012 / Accepted: 2 April 2012 / Published online: 19 April 2012

Abstract Regular surface protein layers (S-layers) from most Gram-positive bacteria and from the ancestral bacterium *Thermus thermophilus* attach to pyruvylated polysaccharides (SCWP) covalently bound to the peptidoglycan through their SLH domain. However, it is not known whether the synthesis of SCWP and S-layer is coordinated enough as to follow a similar pattern of incorporation to the cell wall during growth. In this work we analyse the localization of newly synthesized SCWP on the cell wall of *T. thermophilus* by immunoelectron microscopy. For this, we obtained mutants with a reduced amount of pyruvylated SCWP through mutation of the *csaB* gene encoding the SCWP-pyruvylating activity, and its upstream gene *csaA*, a putative sugar transporter. We hypothesized that CsaA would be required for the synthesis of the SCWP. However, we found that *csaA* mutants showed only a minor decrease in the amount of SCWP immunodetected on the cell walls in comparison with *csaB* mutants, revealing its irrelevance in the process. Complementation experiments of *csaB* mutants with CsaB expressed from inducible promoters revealed that newly synthesized SCWP was homogeneously distributed along the cell wall. Fusions with thermostable fluorescent protein revealed that CsaB was distributed also in homogeneous pattern associated with the membrane. These data support that synthesis of SCWP takes place in disperse and homogeneous form all over the cell surface, in contrast to the zonal incorporation at the cell centre recently demonstrated for SlpA.

Keywords *Thermus* · Secondary cell wall polysaccharide · Surface layer · Pyruvylation · Envelope growth · Outer membrane

Abbreviations

SCWP	Secondary cell wall polysaccharide
SLH	Surface layer homology domain
PG	Peptidoglycan
OML	Outer membrane like of *Thermus thermophilus*

Introduction

The presence of a proteinaceous surface regular array (S-layer) is a common trait among most ancestral and several modern bacterial and archaeal phyla (Sara and Sleytr 2000; Messner et al. 2008). The roles of S-layers differ among organisms, but it is generally accepted that they act in natural environments as protective shields to block access of enzymes and viruses to the subjacent, more critical, envelope layers. Actually, in many "modern" bacteria, S-layers are frequently lost by growth under controlled laboratory conditions. In contrast, in many archaea and in ancestral hyperthermophilic bacteria, S-layers play structural and morphogenetic roles and create specific periplasmic compartments functionally similar to those of Proteobacteria (Beveridge et al. 1997; Castan et al. 2002).

Communicated by M. da Costa.

Electronic supplementary material The online version of this article (doi:10.1007/s00792-012-0448-x) contains supplementary material, which is available to authorized users.

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S-layers are built up by a (glyco)protein (S-proteins) that once purified is able to self-interact to form a crystalline network of identical symmetry and properties as that of the native S-layer. Because lateral S-protein inter-subunit interactions required for self-assembly are so strong, their integration on the cell surface during growth do not depend on mechanism that require lateral diffusion, but on the specific integration of the S-proteins at their final position on the cell. The already existing few studies in which the pattern of S-protein synthesis has been analysed support a diffuse mode of integration of the S-layer on the cell body of the Alfaproteobacteria *Caulobacter crescentus*. This results in a mixing of new and old material over the cell surface. However, on the stalk and at the septum S-protein is incorporated in a zonal fashion and no mixing occurs (Smit and Agabian 1982). In *Bacillus* spp and other Firmicutes newly synthesized S-layer subunits are incorporated as helical bands mainly at septal regions (Gruber and Sleytr 1988). These different models of incorporation could be related with that of the component of the cell surface at which the respective S-protein attaches to.

The mechanisms of S-protein attachment to the cell depend greatly on the nature of the underlying cell envelope layer. In cell wall-less (Gramnegative) Archaea, the S-protein is associated with the cytoplasmic membrane through a hydrophobic amino acid sequence, in such a way that they can be considered as integral membrane proteins (Sleytr and Beveridge 1999), and consequently a "diffuse" integration model is likely. In Proteobacteria, a strain-specific N-terminal domain of the S-protein binds to specific O-chain groups of the lipopolysaccharide (LPS) from the outer membrane (OM). As LPS incorporation follows a diffuse pattern (Bos et al. 2007), the diffuse incorporation of S-proteins (Smit and Agabian 1982) fits with the model. In Firmicutes at least two evolutionarily independent classes of binding domains have evolved within S-proteins (Sara 2001). In *Geobacillus stearothermophilus* and in *Aneurinibacillus thermoaerophilus* DSM 10155, the N-terminal region of their S-proteins, which contains several basic amino acids, is responsible for the binding to specific secondary cell wall polysaccharides (SCWP) through a lectin-like interaction (Sara 2001; Steindl et al. 2002). In contrast, in most *Bacillaceae* the S-protein attachment mechanism involves the binding between a N-terminal triple repetition of a specific conserved domain called SLH (S-Layer homology) (Lupas et al. 1994; Engelhardt and Peters 1998) and SCWP covalently bound to the PG (Ries et al. 1997; Chauvaux et al. 1999; Sara 2001). To the best of our knowledge, there are no reports on the pattern of SCWP incorporation to the cell wall, but it is likely that it should be similar to that of the S-protein for which they constitute the attachment site.

The genus *Thermus,* along with *Deinococcus* and *Meiothermus*, belongs to one of oldest branches of the bacterial phylogeny (Hartmann and Erdmann 1989). The cell envelope of this bacterial group does not fit that of Firmicutes or Proteobacteria well established models. It consists of a complex multilayered structure in which a peptidoglycan (PG) sacculus, whose composition is close to Firmicutes (Quintela et al. 1995), is surrounded by an outer membrane-like structure (OML) that generates a periplasmic space (Castan et al. 2002). Acting as a scaffold for this OML, a hexagonal S-layer exists that is built up by the SlpA S-protein (Castón et al. 1988). Structural, genetic, and biochemical evidences indicate that SlpA represents a peculiar and, most likely, ancestral type of structural protein, with properties shared by both "modern" S-layers and proteobacterial porins (Engelhardt and Peters 1998). Indeed, SlpA is water insoluble, and associates under appropriate conditions in regular structures similar to those built up by bacterial porins instead of the normal hexagonal array exhibited in vivo (Caston et al. 1993). As other OM proteins from primitive bacteria like *Thermotoga* spp, SlpA contains a single SLH domain at its N-terminus instead of the three SLH domains found in S-proteins from Firmicutes (Engelhardt and Peters 1998). This single SLH domain of SlpA is required in vivo for the attachment of the OML envelope to the cell wall (Olabarria et al. 1996), suggesting that it constitutes a primitive form to attach ancestral OMs to the cells. The functional similarity of the OML from *T. thermophilus* to the OM from Proteobacteria has been recently reinforced by the discovery of a homologue of the Omp85 protein family implicated in the insertion of beta-barrel proteins like porins in the OM (Nesper et al. 2008).

In contrast to what it was initially thought, the SLH domain of SlpA does not bind directly to the PG of *T. thermophilus* (Olabarria et al. 1996), but to a SCWP of unknown chemical structure that covers the sacculus. It is likely that such SCWP forms the so-called "intermediate layer" below the OML that was detected both in *Thermus* spp as in *Deinococcus radiodurans* (Cava et al. 2004; Baumeister and Kubler 1978). This layer blocks the access of anti-PG antibodies to the PG, is SDS-insoluble, can be eliminated by treatment with alpha-amylase plus Pronase E, and represents around 14 % of the mass of the SDS-insoluble cell wall fraction (Cava et al. 2004). Its precise composition is not known, but it consists of reducing sugars (74 %), a small amount (4 %) of amino acids, and a 20 % of yet unidentified insoluble components (Cava et al. 2004).

As described for *Bacillus anthracis* (Mesnage et al. 2000), binding of the SLH domain of SlpA to the SDS-insoluble fraction of the *T. thermophilus* cell walls depends on the activity of CsaB, a pyruvylating enzyme encoded by most of the SLH containing microorganisms so far studied. Actually, *T. thermophiluscsaB* deletion mutants show extensive detachment of the OML (Cava et al. 2004)

similar to what was described for mutants expressing a deletion SlpA protein without the SLH domain (Olabarria et al. 1996). These *csaB* mutants contain an almost negligible amount of pyruvic acid (<0.1 μg per mg) in entire SDS-insoluble cell wall fractions compared to that shown by the wild-type strain of *T. thermophilus* (3.8 μg/mg), thus confirming its implication as pyruvylating activity of the SCWP (Cava et al. 2004).

In *Bacillus anthracis*, the *csaB* gene is expressed as part of a two-gene operon (*csaAB*). CsaA is predicted to be a membrane protein with sequence similarities to SpoVB, an enzyme involved in the acquisition of heat resistance by spores, and to putative oligosaccharide transporters (Mesnage et al. 2000). However, at least in *B. anthracis*, CsaA seems to play no relevant role in the synthesis of SCWP. In the genome of *T. thermophilus* HB27 the *csaB* gene (TTC0208) is also preceded by a gene encoding a putative membrane protein that is highly conserved in other isolates from the *Deinococcus-Thermus* phylum, but that has no similarities to CsaA from *B. anthracis*.

In addition to the requirement for CsaB, very little is known about the synthesis of the SCWP. In *B. anthracis*, the best studied model, the participation of TagO, a homologue to glycosyl transferases implicated in the synthesis of teichoic acids has been suggested (Kern et al. 2010), but the inability to isolate *tagO* mutants and the absence of appropriate genetic tools for this bacterium has precluded any further study to confirm these data. It is also unknown whether the synthesis of SCWP takes place at specific sites on the cell wall or in a diffuse way.

In *T. thermophilus*, the SCWP can be labelled with α-SAC (S-layer and cell wall) antiserum (Cava et al. 2004), and a relevant genetic toolbox is available to express proteins in a controlled manner (Cava et al. 2009). Recently, we showed that the new subunits of SlpA are incorporated at the centre of the cell likely following a helical pattern (Acosta et al. 2012). Therefore, we hypothesized that the synthesis of the SCWP could be coordinated with that of SlpA, following a similar pattern of incorporation. Here, we analyse the incorporation of new SCWP from *T. thermophilus* to the cell wall by using a combination of genetics and electron microscopy analysis. Contrary to our expectations, we found that the SCWP seems to be synthesized in a diffuse pattern and not at central points on the cell as SlpA does.

Materials and methods

Bacterial strains and growth conditions

Thermus thermophilus HB27 was a gift from Dr. Koyama. Its derivative HB27c contains a nitrate respiration gene cluster that was acquired by conjugation, allowing this strain to grow anaerobically with nitrate (Ramírez-Arcos et al. 1998). The *E. coli* strain DH5α (*supE44, ΔlacU169, Φ80 lacZΔM15, hsdR17,recA1, endA1, gyrA96, thi-1,relA1*) (Hanahan 1983) was used for genetic constructions. *E. coli* and *T. thermophilus* cells were grown in LB (Lennox 1955) at 37 °C and in *Thermus* broth (TB) (Ramirez-Arcos et al. 1998) at 70 °C, respectively. 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* (Hanahan 1983). When required, genetic selection was carried out on 1.5 % (wt/vol) agar plates with streptomycin (Str; 100 mg/L), kanamycin (Kan; 30 mg/L), and/or ampicillin (Amp; 100 mg/L). For controlled expression of genes in *T. thermophilus* under the control of the promoter of the nitrate reductase gene, cells were grown at 70 °C under stirring (150 rpm) in flask filled 1:5 of the volume with TB. At an optical density at 550 nm (OD_{550}) around 0.3, potassium nitrate (40 mM) was added, stirring was stopped to decrease the amount of soluble oxygen, and the incubation was maintained for the required time.

Isolation of mutants

For the isolation of nonpolar *csaA* deletion mutants, we followed a pop in–pop out strategy as described (Blas-Galindo et al. 2007). For this, we amplified by PCR DNA regions located immediately upstream (oligonucleotides UKdirecto2 and UKreverso2, Table 1) and downstream (oligonucleotides csaBdirecto2 and csaBreverso2, Table 1) of the *csaA* gene. By using the restriction enzymes *Eco*RI, *Xba*I, and *Hin*dIII sites included in the sequence of the oligonucleotides, we constructed the suicide plasmid pS18Δ*csaA* that was used to transform *T. thermophilus* HB27, selecting on TB plates with streptomycin. A group of small-sized transformant colonies that grew after 24 h at 70 °C, were co-inoculated in 10 ml of TB medium and incubated under stirring for 2 h at 70 °C to allow the back-recombination of the plasmid. Aliquots were spread onto TB plates without any antibiotic and further incubated until colonies developed. As the *rpsL1* allele confers a dominant streptomycin-dependent phenotype (Blas-Galindo et al. 2007), only those colonies in which this allele was deleted by back-recombination grew. The Str-sensitive clones were then analysed for the absence of the *csaA* targeted gene by PCR with primers check-csaA-csaB and check-csaA-UK (Table 1).

For the isolation of *ΔcsaB* mutants, the strategy with pS18a derivatives failed, so we used derivatives of the suicide pK18 plasmid (Laptenko et al. 2006) that confer kanamycin resistance. The construction of the mutagenic plasmid pK18Δ*csaB* was carried out in two steps. We first cloned a 3-kbp fragment, amplified by PCR from genomic

Table 1 Oligonucleotides used in this work

Name	Sequence (5′ > 3′)	Purpose
UKdirecto2	AAAGAATTCGGAGAAGGTCTACCTCGTGG	PCR upstream *csaA*
UKreverso2	CTTTCTAGACTCACGCTGCCCCCATCC	PCR upstream *csaA*
csaBdirecto2	ATTCTAGACCTGGTAAGGAGGCTTAGG	PCR downstream *csaA*
csaBreverso2	TTTAAGCTTAGGACCTCAGGGCCTTCG	PCR downstream *csaA*
check-csaA-csaB	TGGTGGCGTCCTGCAGAA	Analysis of *ΔcsaA* mutants
check-csaA-UK	GGAAGGCATCCTGGTCCTCTA	Analysis of *ΔcsaA* mutants
csaEco1a	CCGGAATTCTCAGGCTCTTCAGCC	PCR of *csa* cluster and *ΔcsaB* checking
csaSal1a	ACGCGTCGACCGCCAGCGCCTCC	PCR of csa cluster and *ΔcsaB* checking
csaBXba2	GCTCTAGACACCATGCCCTAAAGC	Divergent PCR to get pK18Δ*csaB*
csaB Xba3a	GCTCTAGACAAGGCCCTGAGGTC	Divergent PCR to get pK18Δ*csaB*
csaNde	CTTAGCATATGGTGGTCG	Construction of pMKE*csaB*
csaHind	AACAAGCTTGCCTCAGCCGCGC	Construction of pMKE*csaB*
csaBNdedir	AAAAACATATGTAAGGAGGCTTAGGGCATGGTGGTCGGC	Construction of CsaB–sGFP fusion
csaBnsEcorev	TTTTTGAATTCGCCGCGCCCGGAGCCTTTGATGGG	Construction of CsaB–sGFP fusion

Restriction sequences for *Eco*RI, *Xba*I, *Xba*I and *Hin*dIII are underlined

DNA of the HB27 strain with primers csaEco1a and csaSal1a (Table 1), between the *Eco*R1 and *Sal*I sites of plasmid pK18. In a further step, we carried out divergent PCR on this plasmid with primers csaBXba2 and csaBXba3a (Table 1) and further re-ligation after digestion with *Xba*I, recognition sequence of which was included in the oligonucleotides. Plasmid pK18Δ*csaB* was used to transform the nitrate-respiring HB27c strain, and selection on TB-kanamycin was carried out. Clones with the inserted plasmid were grown in liquid TB without antibiotic for around 20 generations, and clones sensitive to kanamycin were isolated and analysed by PCR for the absence of the *csaB* gene with oligonucleotides csaEco1a and csaSal1a (Table 1). Isolation of the *csaB::kat* insertion mutant was described previously (Cava et al. 2004).

A polar *csaA*::*kat* mutant was also constructed on the HB27 strain by insertion of the suicide plasmid (pK18*csaA*) carrying *csaA* gene internal fragment encoding from positions 229 to 470 of the protein. This vector was constructed from plasmid pK18ΔcsaB by removing a 1,246 kbp fragment by *Hin*dIII digestion. The plasmid was used to transform the wild-type strain and clones resistant to kanamycin were analysed by PCR to confirm the insertion.

Controlled expression of csaB

For the expression of *csaB* gene in the Δ*csaB* mutant in complementation experiments, the coding sequence was amplified by PCR with oligonucleotides csaNde and csaHind. The PCR fragment was cloned between the *Nde*I and *Hin*dIII sites of *E. coli–T. thermophilus,* shuttle vector pMKE1 using the restriction sites included in the primers. From this plasmid, the *csaB* gene can be expressed under the control of the promoter for the respiratory nitrate reductase, whose transcription is activated by nitrate under low oxygen pressure (Moreno et al. 2003). Once induced, samples were taken at different times, and SDS-insoluble cell wall fractions were prepared and analysed.

Expression of fluorescent CsaB–sGFP fusions

For the construction of fusion proteins, the coding sequence of *csaB* was amplified by PCR with primers csaBNdedir and csaBnsEcorev (Table 1). The PCR product was digested with *Nde*I and *Eco*RI and cloned into pMKPnarsGFP (Cava et al. 2008). From this plasmid, a CsaB–sGFP fusion can be expressed under the control of the nitrate reductase promoter as above.

Isolation of SDS-insoluble cell wall fractions

The SDS-insoluble cell wall fraction was isolated after 8-h periods of boiling in 6 % (w/v) sodium dodecyl sulphate (SDS) of cells, followed by repeated steps of centrifugation (80,000*g*, 15 min, 30 °C) and washing in water to get rid of the remaining SDS (Pisabarro et al. 1985).

Optical, confocal and electron microscopy

Phase contrast images of cells were obtained in an Axioscop Zeiss microscope coupled with a Coolsnap FX camera. For fluorescence and confocal microscopy, 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.

For electron microscopy the method described by (de Pedro et al. 1997) was followed. In essence, purified cell walls were adhered to carbon-pioloform-coated grids and immunolabelled with diluted αSAC antisera for 60 min. After washing, bound antibodies were detected with 5 or 10 nm gold–protein A conjugates, and the presence of the cell walls was further contrasted by staining with 1 % (wt/vol) uranyl acetate. Microscopic observations were performed on a Philips CM10 transmission electron microscope at an acceleration voltage of 40 or 60 kV.

Bioinformatics

Sequence comparisons of the CsaB proteins were carried out at the website http://www.ncbi.nlm.nih.gov/entrez/ using the BLAST program (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST/).

Results

The *csa* operon of *T. thermophilus*

The CsaB protein of *T. thermophilus* HB27 is encoded by the chromosomal gene TTC0208. Previous work from our laboratory showed that CsaB is required for the pyruvylation of the cell wall of this organism (Cava et al. 2004). A detailed analysis of its genetic context reveals the presence of a gene (TTC0207) encoded in the complementary strand immediately downstream of *csaB*. This fact indicates that *csaB* has to be transcribed as the last gene from an mRNA. Overlapping 16 nucleotides with the 5′ coding sequence of *csaB* is another gene (TTC0209) most likely co-expressed in the same transcript, that will be called *csaA* thereafter. In a similar way, the ATG start codon of *csaA* overlaps with the stop codon from the preceding gene (TTC0210). A 100 % homologue of the protein encoded by this gene from the strain *T. thermophilus* HB8 has been recently characterized biochemically and structurally (Tomoike et al. 2011). The 211 amino acids-long protein has a cytidine-specific kinase activity, apparently implicated in the salvage pathway of this nucleoside. Because of its conserved clustering, and likely co-expression with *csaA* and *csaB*, we will call this gene *csaU* thereafter. Upstream of *csaU*, a putative Rho-independent transcriptional terminator sequence exists, supporting that *csaU-csaA-csaB* may be transcribed as a tricistronic mRNA. A similar clustering of homologues is encoded in several strains from the phylum *Thermus-Deinococcus* (Fig. 1), supporting that the whole cluster is implicated in a related function in all of them.

Translation of *csaA* renders a 566-amino acid protein for which 8 or 7 transmembrane α-helices are predicted depending on whether or not the N-terminal one is processed as signal peptide. Homologues to CsaA with 99 % identity are found in the strains HB8 and SG0.5JP17-16 of *T. thermophilus*. In other *Thermus* spp, sequence identities

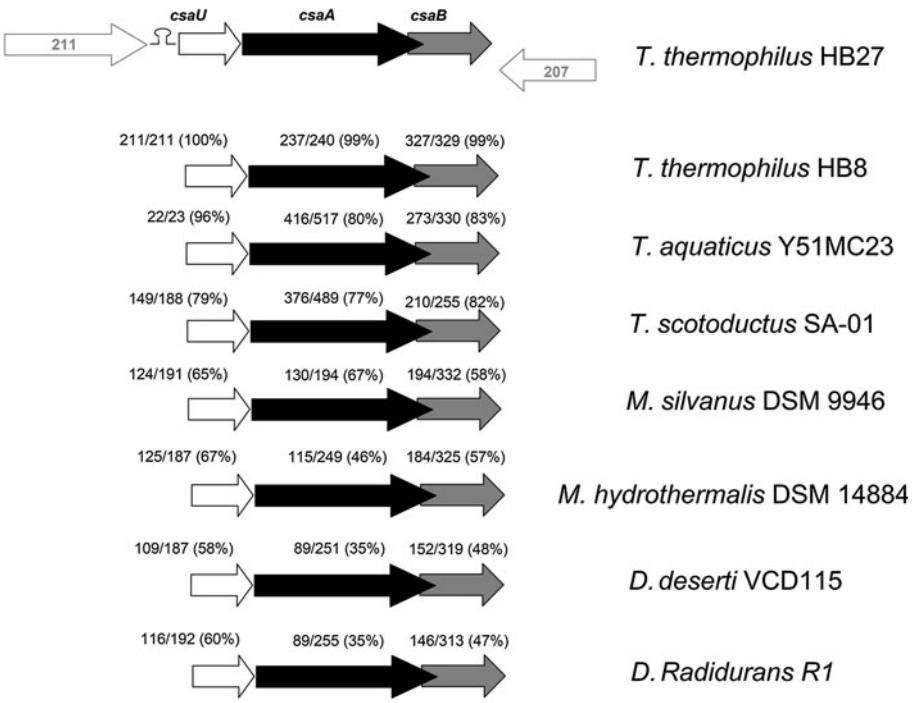

Fig. 1 The *csa* cluster in the *Deinococcus-Thermus* phylum. Figure shows the *csaU*, *csaA* and *csaB* genes as *arrows*. *Numbers* indicate identity and (similarity) percentages. Organisms: *Thermus thermophilus* HB27 and HB8, *Thermus aquaticus* Y51MC23, *Thermus scotoductus* SA-01, *Meiothermus silvanus* DSM9946, *Marinithermus hydrothermalis* DSM14884, *Deinococcus deserti* VCD115, *Deinococcus radiodurans* R1

of CsaA homologues rank between 77 % (*T. scotoductus* SA-01) and 80 % (*T. aquaticus* Y51MC23 and *Thermus* sp. CCB_US3_UF1). In other genus of the same phylum, sequence identity ranges from 57 % (*Meiothermus silvanus* DSM 9946) to 37 % (*Deinococcus* spp.). Outside this phylum, CsaA homologues (25–35 % of identity) are found among Firmicutes like *Desulfotomaculum* spp. or *Selenomonas* spp, a Firmicute that stains negative in the Gram reaction.

Despite the fact that CsaA protein has no detectable conserved domains (BLASTp, NCBI server), its membrane character and the strong association with *csaB* suggested a putative role in the transport of sugars during the synthesis of secreted polysaccharides, that could likely be the pyruvylated SCWP to which SlpA binds. In fact, at least one of the CsaA homologues (27 % of identity) from *Ornithinibacillus* sp. TW25 has been annotated as a "polysaccharides export" protein.

The role of *csaA*

In order to check if *csaA* was relevant for the synthesis of the SCWP, we isolated two types of mutants: a *ΔcsaA* deletion mutant and *csaA*::pK18 insertion ("Materials and methods"). As shown in Fig. 2, the insertion of pK18 into *csaA* produced a phenotype similar to that described for *csaB* mutants, in which the OML was not attaching properly to the cell wall leading to the production of several multicellular bodies (MBs) per optical field, whereas in the wild-type strain these structures were not detected. MBs are defined as clonal groups of cells surrounded by a common OML (Cava et al. 2004). Such phenotype was expected because the insertion, in addition to deleting 97 C-terminal amino acids from CsaA, interrupts transcription and avoids expression of *csaB*. In contrast, *ΔcsaA* deletion mutants, in which the transcription of *csaB* should not be modified, did not produce MBs but a slightly filamentous phenotype (Fig. 2a). These data support that CsaA could play a role in the synthesis of the cell wall, but is not a major player in the expression or modification of the SCWP to which the SlpA protein binds.

Effects of CsaA inactivation on the amount of SCWP

To confirm the observations above, we prepared crude cell walls from the wild-type strain and from its derivatives *ΔcsaA* and *csaB::kat*, and carried out immunoelectron microscopy with αSAC. As shown in Fig. 3a, a homogeneous labelling of the cell wall was observed in the wild-type strain and in the *csaB::kat* and *ΔcsaA* mutants. As a way of detecting the amount of SCWP present on the cell wall, counts of gold particles per surface unit were carried out on a minimum of 40 cells in three independent experiments. As it can be seen in

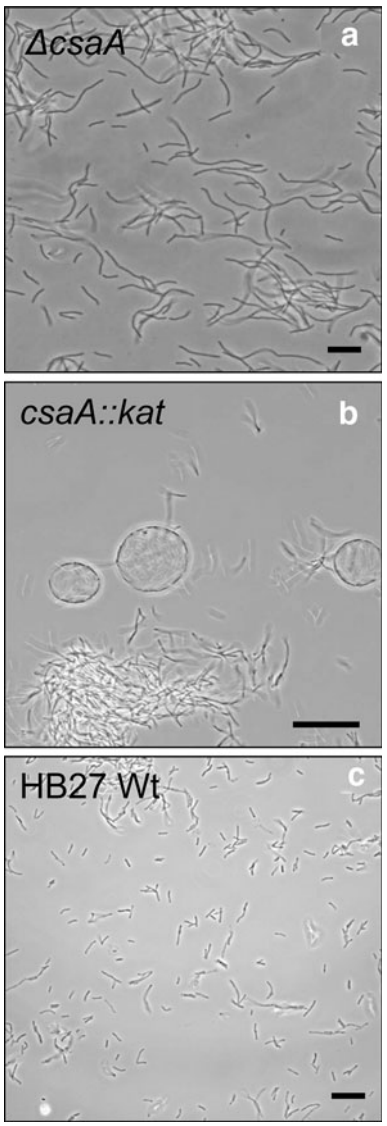

Fig. 2 Morphology of *csaA* mutants. Phase contrast images of cultures of the *ΔcsaA* (**a**) and the insertion *csaA::pK18* (**b**) mutants of *T. thermophilus* HB27 grown in TB medium up to an OD_{550} of 1. *Bars* correspond to 5 μm

Fig. 3, a reduction in the number of particles of around 25 % was detected in the *csaB::kat* mutant. In the *ΔcsaA* mutant, a decrease in the labelling was also detected, but smaller than that observed for the *csaB::kat* mutant. In contrast to our initial hypothesis, these data suggest that absence of CsaA has a minor effect on the synthesis of SCWP in *T. thermophilus* HB27, supporting the existence of an alternative pathway involved in this process.

Localization of newly synthesized SCWP on the cell walls

To analyse the pattern of incorporation of new SCWP to the PG, we expressed CsaB in a controlled manner in a

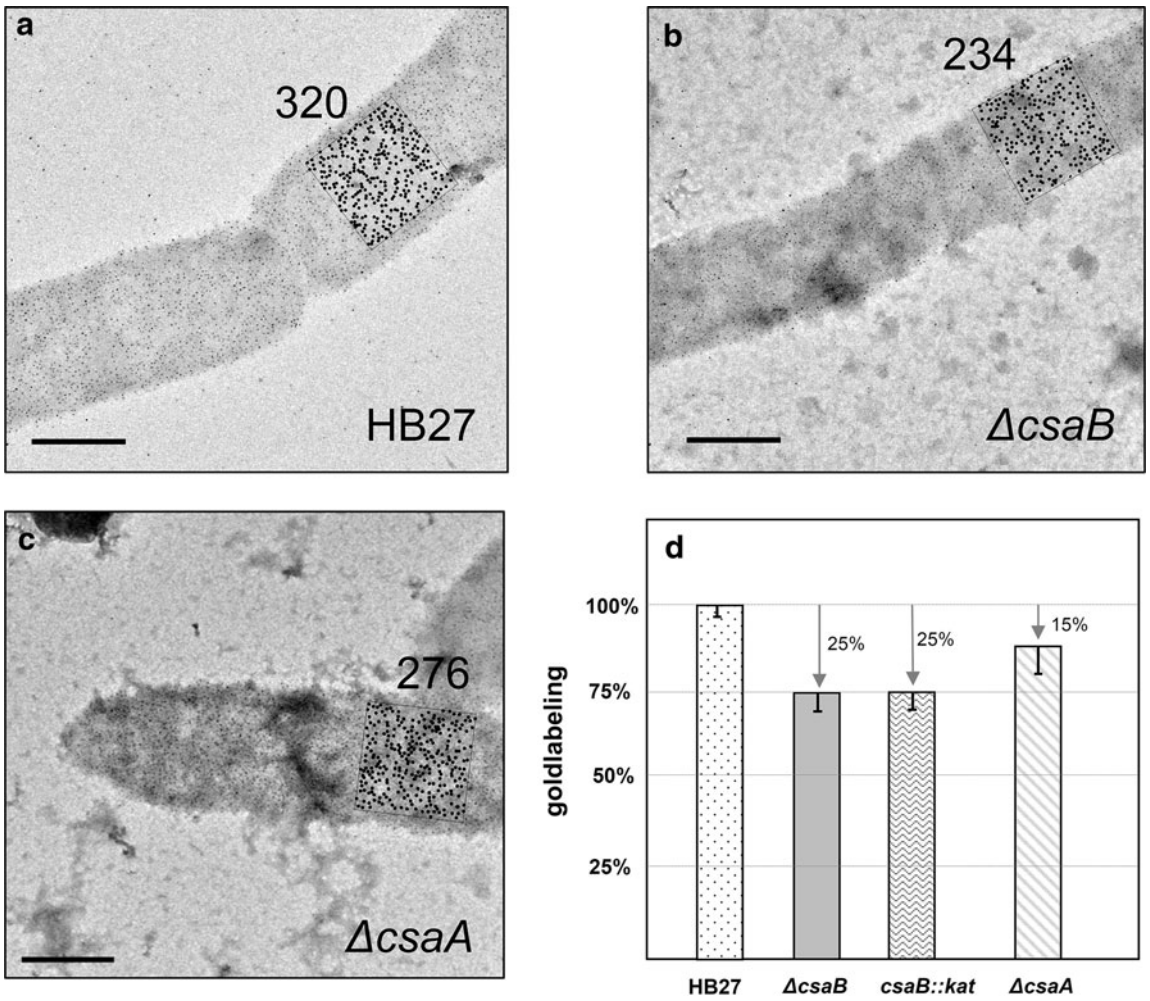

Fig. 3 Immunolabelling of whole cell walls from *T. thermophilus* HB27. Cell walls from the wild-type strain HB27 (**a**), the *csaB::kat* (**b**), and *ΔcsaA* (**c**) mutants were immunolabelled with α-SAC as described in "Materials and methods". **d** The number of gold particles detected per unit of cell surface of at least 40 cells in three independent experiments was counted and represented as percentage to that of the wt. *Bars* indicate standard deviations. The values for the *ΔcsaB* derivative of the strain HB27c is also shown. Gold particles in the selected areas have been highlighted in the images **a**–**c** to facilitate their observation. Original images are included as Supplementary Material

csaB null mutant, and followed the accumulation of SCWP on the cell walls versus the time by immunolabelling. For this, we used the pMKECsaB plasmid, in which *csaB* is expressed under the control of the Pnar promoter. As this plasmid requires kanamycin for selection and a host containing the gene cluster for nitrate respiration for expression, we had to isolate a marker-free *ΔcsaB* mutant from the HB27c strain ("Materials and methods"). This is a derivative of HB27 to which the capability for anaerobic growth with nitrate was transferred by conjugation, allowing the controlled transcription from the Pnar promoter (Moreno et al. 2003). As expected, the entire cell wall of the *ΔcsaB* mutant showed a decrease in SCWP immunolabelling with respect to the wild type similar to that shown by the *csaB::kat* mutant (Fig. 3d).

As shown in Fig. 4, under aerobic conditions when the Pnar promoter is repressed, crude walls from cells transformed with pMKECsaB showed similar density of α-SAC immunolabelling as those carrying the empty plasmid (time 0). After 30 min of induction, walls from cells carrying pMKECsaB showed an increase (12 %) in the amount SCWP detected. After 1 h, the increase in labelling reached its maximum (around 50 %), and started to decrease at longer times (2 and 4 h). After overnight incubation, the amount of SCWP detected was close to that in mutant cells carrying the empty plasmid.

When the patterns of labelling along the cell walls (e.g. "new" SCWP) were analysed in greater detail in 30 sacculi in three independent experiments, no significant differences in the distribution were found between induced

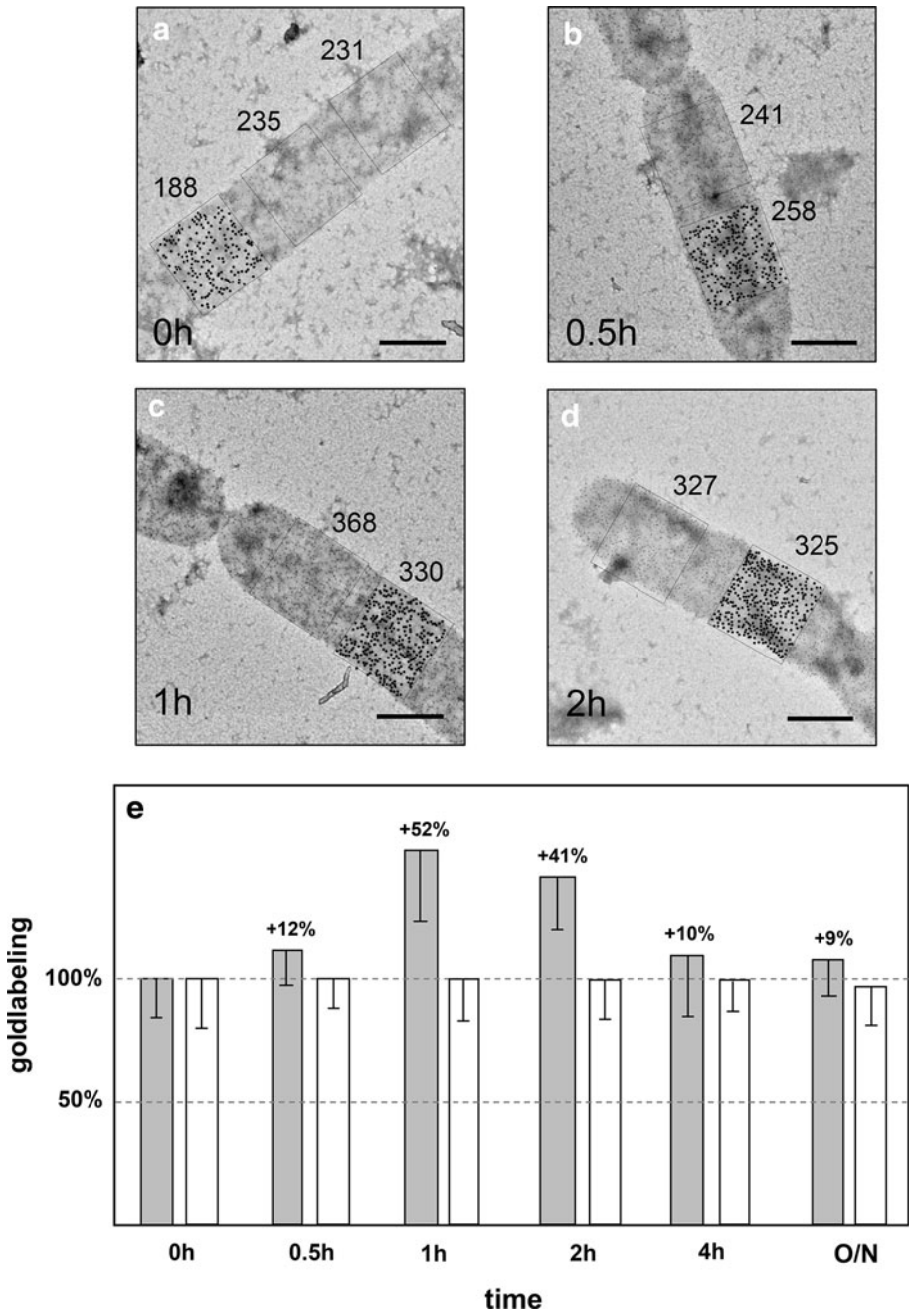

Fig. 4 Controlled expression of CsaB in *ΔcsaB* mutants. **a** Cells of *T. thermophilus* HB27c *ΔcsaB* transformed with the pMKECsaB (*grey bars*) or the control pMKE1 (*white bars*) plasmids grown aerobically (time 0), were subjected to nitrate respiration conditions to induce the expression of the Pnar promoter. At the indicated times samples were collected, and the entire cell wall purified and subjected to immunolabelling with α-SAC. Representative images of the cells at each time are presented in **a**–**d** with the areas used for counting gold particles. **e** Presents the data obtained from a minimum of 30 cell walls per point. *Bars* indicate standard deviations. Gold particles in the selected areas have been *highlighted* in the images **a**–**c** to facilitate their observation. Original images are included as Supplementary Material

(30 min to 2 h) and control cells. Actually, the increment in SCWP labelling detected was basically uniform when different sections of the cells were analysed, showing that either whole SCWP synthesis or its pyruvylation takes place homogeneously on the cell wall, and not at a central location as we expected from the pattern of incorporation of SlpA (Acosta et al. 2012).

CsaB is associated with the cell membrane

If the above conclusion was correct, a homogeneous distribution of the CsaB protein in the cytoplasm was expected. In order to confirm this, we constructed a fusion between CsaB and a thermostable form of the green fluorescent protein (sGFP) that can be used in *T. thermophilus* (Cava et al. 2008). Unexpectedly, constitutive expression of the CsaB–sGFP protein fusion lead to the accumulation of fluorescence at the cell boundary, as shown in the optical sections of Fig. 5a and in the corresponding inset. This apparent membrane-associated expression was not concentrated in specific regions of the cell, but uniformly distributed over the entire surface. A control expressing only sGFP showed fluorescence distributed on the whole cell (Fig. 5b).

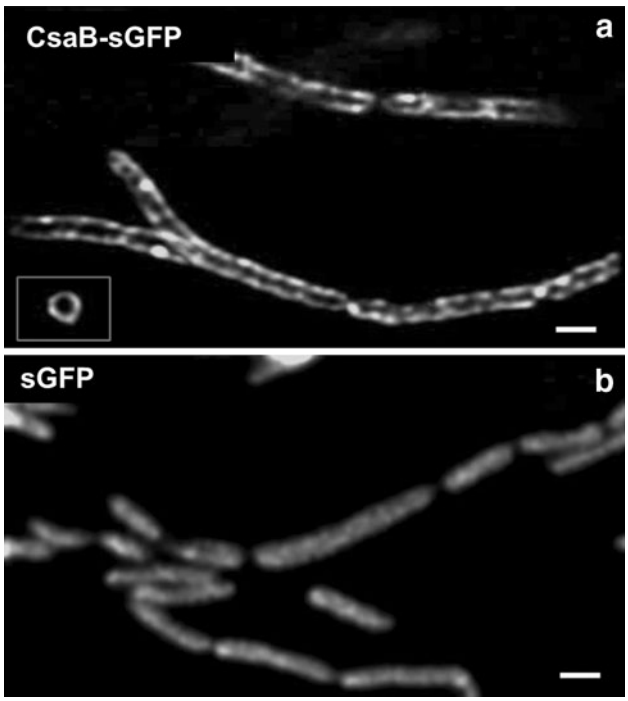

Fig. 5 CsaB localizes to the membrane. A CsaB–sGFP fusion was expressed from plasmid pMKPnqosGFP-csaB in *T. thermophilus* HB27, and the presence of the fluorescence identified by confocal microscopy. **a** Optical sections showing the distribution of the fluorescence to the membrane. A detail of a transversal section is shown. **b** Control expression of sGFP. *Bars* represent 0.5 μm

Discussion

The cell envelope of *T. thermophilus* has a complexity similar to that of Proteobacteria but contains elements closer in composition to Gram-positives. The outer membrane like (OML) is associated to a scaffold of regularly arrayed protein SlpA that also acts as a bridge to attach the OML to a pyruvylated SCWP layer, which is covalently bound to the PG (Cava et al. 2009). In a recent work, we showed that the synthesis of the OML, and particularly of the SlpA protein, takes place at a central part of the cell during cell elongation (Acosta et al. 2012). Based on the SCWP-SlpA attachment, we hypothesized that the patterns of synthesis and incorporation of SCWP could be similar to that of SlpA. However, we show here that in contrast to our expectations the incorporation of the SCWP takes place following a basically "diffuse" model.

The role of *csa* genes in the synthesis of SCWP

The conservation of the clustering of the *csaB* gene with *csaA* and *csaU* in the whole *Deinococcus-Thermus* phylum, suggested their implication in a common biosynthetic pathway, likely related or active in the synthesis of the SCWP layer common to the phylum. CsaU catalyses the phosphorylation of cytidine with ATP to render CMP (Tomoike et al. 2011). Therefore, is tempting to speculate on a role of this protein providing a surplus of cytidine nucleotides required to stimulate growth as activators of sugars for the synthesis of external polymers, like in the case of the CDP-ribitol intermediate in the synthesis of teichoic acids in *Streptococcus pneumoniae* (Baur et al. 2009).

The integral membrane protein character of CsaA suggested its implication in the synthesis of SCWP. It is noteworthy that *csaB* genes from most Gram-positives are also preceded by genes encoding membrane proteins. In *B. anthracis* the corresponding protein was also named CsaA, and was homologous to *Bacillus subtilis* SpoVB, a protein required for acquisition of heat resistance by the spores, and to sugar transporters of Gram-negatives (Mesnage et al. 2000). *T. thermophilus* CsaA shows low similarity to this protein of *B. anthracis*, but shows up positive BLAST similarity results with membrane enzymes involved in the biosynthesis of PG, like undecaprenyldiphospho-muramoylpentapeptide-*β*-*N*-acetyl-glucosaminyl transferase and proteins annotated as polysaccharide export proteins. These comparisons suggested a putative implication of CsaA in the synthesis of the SCWP, but our data did not support this. Actually, *csaA* mutants have no major morphological alterations except when polar mutations affecting CsaB were used (Fig. 2). However, a small decrease (15 %) in the amount of labelling with αSAC was detected, suggesting either that the deletion of *csaA* modified the cell wall surface limiting access to the antibodies, or that CsaA is actually involved in SCWP synthesis but an alternative pathway for SCWP exists and is able to compensate for CsaA inactivation. Another possibility of explaining the observed decrease in labelling could be related with a decrease in the levels of CsaB as a consequence of the *csaA* mutation. In fact, as the ribosome binding site of *csaB* is located inside *csaA*, the translational coupling that should exist between these two overlapping genes could be partially affected despite conservation of the RBS in the deletion. In any case, our data support that *csaA* is not essential for SCWP synthesis. Noteworthy, in *B. anthracis* the *csaA* gene is also apparently irrelevant in the synthesis of the SCWP despite being cotranscribed with *csaB* (Mesnage et al. 2000), whereas a homologue to TagO (BAS5050), an enzyme implicated in the synthesis of teichoic acid in many Gram-positives has been recently shown to be essential for SCWP synthesis (Kern et al. 2010). In fact, TagO inhibition with tunicamycin produces S-layer deficiencies, supporting its implication in SCWP synthesis (Kern et al. 2010). A homologue to TagO is encoded in the genome of *T. thermophilus* HB27 (TTC0497). This gene is annotated as a putative glycosyl transferase and is clustered with genes encoding putative

UDP-*N*-acetylglucosamine 2-epimerase (TTC0498) and uracil phosphoribosyltransferase (TTC0496), enzymes that could also be implicated in the synthesis of extracellular polysaccharides like the SCWP. These data indicate that the TTC0496-0498 set of genes could be responsible for SCWP synthesis. In any case, and due to the apparent irrelevance of *csaA* in the process, our only way to study the synthesis of the SCWP was the utilization of *csaB* mutants.

Disperse synthesis of SCWP

Cells defective in CsaB still synthesize a non pyruvylated form of SCWP for which the SLH domain of SlpA has lower affinity, leading to the formation of MBs as those shown in Fig 2b (Cava et al. 2004). However, we only detected a moderate (25 %) decrease in the amount of immunolabelling with α-SAC of the cell walls of the *csaB* mutants, supporting the existence of antibodies in this rabbit antiserum that recognize other components of the cell walls. As α-SAC was raised against the SlpA protein and the cell wall fragments bound to the protein after cell wall digestion with muramidase (Cava et al. 2004), it contains antibodies that recognize the SCWP and PG fragments containing Dala–Dala muropeptides (our own unpublished work).However, the PG of *csaB* mutants was accessible to antibodies raised against the peptidoglycan of *E. coli* only after treatment of the cell walls with α-amylase and pronase E (Cava et al. 2004), supporting the presence of a layer of nonpyruvylated SCWP that blocked the access to the peptidoglycan. In consequence, it is likely that the background immunolabeling of the cell walls from *csaB* mutants corresponds to the detection of these nonpyruvylated SCWP. Despite this background, we observed the expected increase in the amount of labelling upon expression of the *csaB* gene, allowing to recover and even to surpass the labelling of the wild type. As the expression from this plasmid is usually higher than that of the corresponding protein in the wild-type strain (Moreno et al. 2003), these data suggest that the biosynthetic apparatus implicated in SCWP synthesis limits the amount of the pyruvylated form of this compound incorporated into the cell wall independently of the amount of CsaB available. Moreover, the decrease in labelling detected after 2 h of induction, when the Pnar promoter decreases its expression, suggests that CsaB is either inhibited or degraded by unknown mechanisms and, on the other hand, that SCWP is a dynamic component of the cell wall that can be incorporated and also degraded during growth. In this sense, it is important to know that identification of the sugars that are pyruvylated by CsaB homologues on SCWP from Gram-positives has been elusive until recently because only a small fraction of the SCWP is actually pyruvylated (Kern et al. 2010). Therefore, pyruvylation seems to be a well-regulated process in the synthesis of the cell wall, and the organisms probably have developed quality control mechanisms to check the integrity of the SCWP layer and regulate their amount, in a similar way to those existent for PG (den Blaauwen et al. 2008).

The distribution of the labelling after CsaB induction did not show any specific pattern, and gold particle numbers per surface unit were basically identical along the major axis of the cell, showing that the synthesis of pyruvylated SCWP followed a "disperse" and not a "zonal" model. This "disperse" model of synthesis does not fit with the highly localized incorporation of SlpA to the envelope, but is similar to the growth models proposed for PG sacculi in bacillar bacteria such as *E. coli* and *Bacillus subtilis* (Harold 2007). In the latter, it has been recently shown that the MreB filament protein seems to organize circumferential motions of the PG synthesis machinery at several points along the cell body (Garner et al. 2011). As this mechanism seems to be conserved—actually *T. thermophilus* has a MreB homologue—it is reasonable to think that SCWP could be incorporated following a similar pattern in *T. thermophilus*, and likely in other bacteria.

In the above scenario, the localization of CsaB to the membrane around the whole cell body fits with a diffuse model of SCWP synthesis. Accordingly, we speculate that CsaB, predicted as a soluble protein, attaches to membrane components of a SCWP synthesis apparatus leading to pyruvylation of precursors once bound to the membrane transporter, and that this apparatus is coordinated with PG synthesis machinery.

Acknowledgments This work has been supported by grant BIO2010-18875 from the Spanish Ministry of Science. An institutional grant from Fundación Ramón Areces to CBMSO is acknowledged. A FPI fellowship to F. Acosta is also acknowledged. Financial support to the Spanish National Network for Extremophilic Microorganisms (BIO2011-12879-E) is also acknowledged.

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