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Multiple Regulatory Mechanisms Act on the 5′ Untranslated Region of the S-Layer Gene from *Thermus thermophilus* HB8

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The role of the 5′ untranslated region (5′UTR) of the S-layer gene from *Thermus thermophilus* was analyzed through the isolation of Δ5′UTR mutants. In these mutants the half-life of *splA* mRNA was strongly reduced and *slpA* transcription was no longer subjected to growth phase-dependent repression. Overproduction and detachment of the external envelopes of the mutants were observed in stationary phase.

The envelope of the extreme thermophile *Thermus thermophilus* HB8 is a multilayered structure consisting of cytoplasmic membrane, peptidoglycan (13), a thick amorphous layer (intermediate layer), the S-layer, and an EDTA-sensitive material that occludes the access of antibodies to the S-layer (reference 3 and our unpublished results).

In vitro evidence suggested that overlapping transcriptional and translational controls were acting on the 5′ untranslated region (5′UTR) of the S-layer protein-encoding gene *slpA* (7). This 127-bp region includes inverted repeats flanking a DNA sequence of high bending potential that could act as a binding site for putative transcription factors (5). In addition, the leader mRNA transcribed from this 5′UTR has the potential to form a highly folded structure (references 5 and 7 and our unpublished results) similar to that described for S-layer genes from gram-positive bacteria and for the OmpA-encoding gene of *Escherichia coli* (4). Northwestern blots suggested that this folded structure also could be implicated in putative translational autoregulation (7).

However, this complex regulation was deduced from a combination of in vitro assays and a series of experiments performed in *E. coli* well below the temperature at which *T. thermophilus* grows (70 to 75°C). Consequently, it became necessary to test in vivo, such as a regulation through the isolation and analysis of mutants in which the 5′UTR was deleted.

Construction of mutants with a modified *slpA* promoter region. The sequence of the *slpA* promoter and its 5′UTR is shown in Fig. 1A. A deletion derivative containing positions −67 to +2 (7) was used to express a bicistronic fusion between a gene encoding a thermostable resistance to kanamycin (*kat*) and the S-layer gene *slpA* (Fig. 1B). This construct was used to replace the wild-type gene by selecting for kanamycin resistance (9). The *kat* gene was subsequently deleted from this mutant by transformation with the appropriate plasmid followed by a double cycle of negative selection with ampicillin. The sequence of the *slpA* promoter from one of the kanamycin-sensitive mutants isolated (HB8ΔUTR1) is shown in Fig. 1C.

Role of 5′UTR in the stability of *slpA* mRNA. The presence of 5′UTR mRNA sequences preceding S-layer genes has been proposed to stabilize the mRNA of S-layer genes from gram-positive bacteria (2). To explore if this was the case in *T. thermophilus*, the half-lives of the *slpA* mRNAs from the wild-type and Δ5′UTR mutants were investigated by Northern blotting. Detection of *slpA* was achieved with the oligonucleotide OslpA-1 (5′-CAGGGCCTCCACGGC-3′), which was labeled and revealed with Gene Images 3′ oligolabeling and the enhanced chemiluminescence (ECL) detection kits (Amersham-Pharmacia Biotech), respectively.

As shown in Fig. 2A, the *slpA* gene from each of the Δ5′UTR mutants was transcribed in mRNAs of the expected sizes. The experiment revealed that the mRNA from both mutants was not detected after 5 min, whereas that of the wild type was still high after 15 min. Thus, a decrease in half-life of a minimum of approximately sevenfold was the consequence of the Δ5′UTR mutation.

Interestingly, the amount of mRNA in the mutants and the wild type was similar at time zero, showing that a higher transcription rate compensates for the faster mRNA degradation. In fact, the amount of S-protein produced by the wild type and the mutants was similar (data not shown), suggesting that deletion of the leader mRNA does not affect its translational efficiency, at least in these conditions (exponential growth).

Role of 5′UTR in growth-dependent repression of *slpA*. The transcription of *slpA* was followed along the growth of cultures of the wild type and the Δ5′UTR mutants. As shown in Fig. 2B, transcription of *slpA* in the wild type decreased as the cells reached the stationary phase, being undetectable after 24 h of growth (Wt, lane 4). In contrast, transcription of *slpA* remained constant in both mutants after 24 h of growth. Thus, a growth phase repression of *slpA* was dependent on its 5′UTR.

Phenotypic effects of 5′UTR deletion. Wild-type cells of *T. thermophilus* HB8 appear as short filaments and single cells in stationary phase (Fig. 3A). However, a high proportion of spherical bodies filled with cells (multicellular bodies [MBs])

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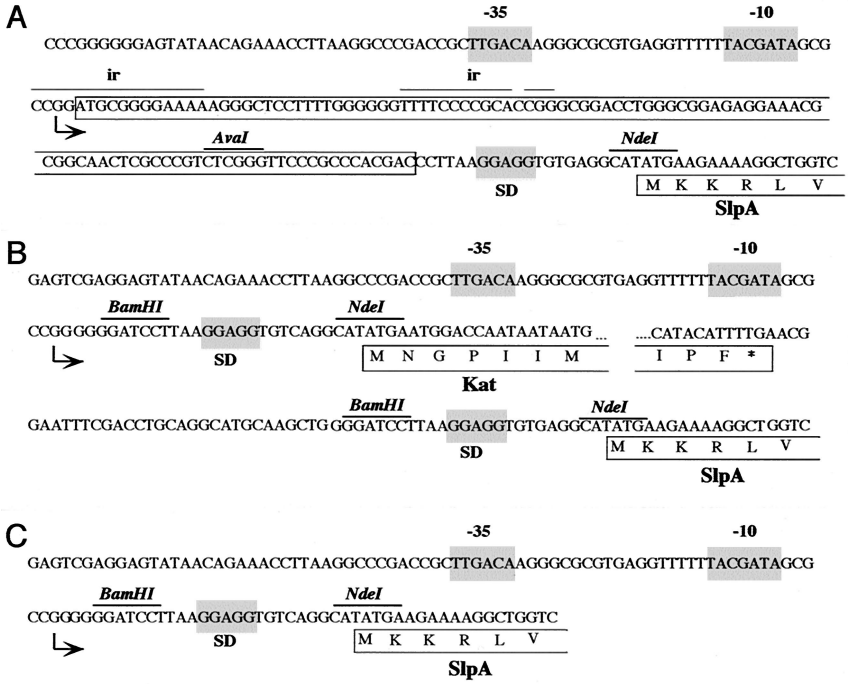

FIG. 1. Sequence of the *slpA* promoter region in the wild type and the Δ5′UTR mutants. The sequences of the *slpA* promoter of the wild type (A) and the HB8ΔUTRK1 (B) and HB8ΔUTR1 (C) mutants are shown. Gray boxes indicate the consensus −35, −10, and ribosome binding site (SD) sequences. The thick arrow indicates the transcription and the thin lines label the inverted repeats (ir) in the wild-type promoter. The N- and C-terminal amino acids of the Kat and SlpA proteins are shown under the sequences. The box in the wild-type promoter labels the 5′UTR sequence deleted in the mutants.

was observed in cultures of both Δ5′UTR mutants (Fig. 3B). These MBs accumulated and grew in size as the cultures reached the stationary phase (data not shown).

The MBs were very sensitive to mechanical disruption. To get thin sections suitable for electron microscopy, cells from the HB8ΔUTR mutant were fixed in 1% glutaraldehyde and carefully sucked into cellulose capillaries (internal diameter, 200 μm) as described previously (8). After dialysis against 1% glutaraldehyde in phosphate-buffered saline, capillaries were infiltrated with 30% (vol/vol) dimethyl-formamide for 15 min, frozen in liquid propane (−185°C), and freeze-substituted for 12 h in 0.5% (wt/vol) OsO_4 in acetone. Once at room temperature, they were washed with acetone and infiltrated in acetone-Epon and further in Epon alone. Upon polymerization (overnight at 65°C), thin sections were prepared, poststained in

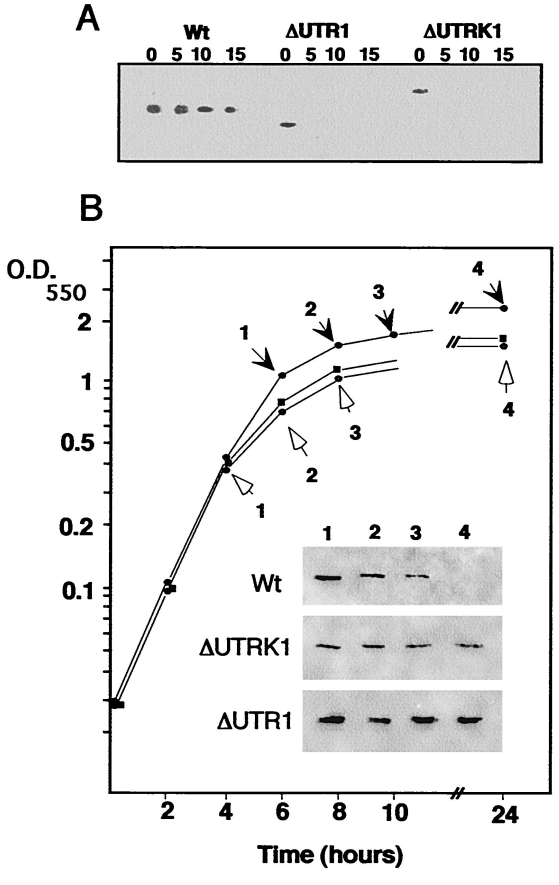

FIG. 2. Expression of the *slpA* gene in Δ5′UTR mutants. (A) The stability of the *slpA* mRNA from the wild type (Wt) and the HB8ΔUTRK1 (ΔUTRK1) and HB8ΔUTR1 (ΔUTR1) mutants is analyzed. Exponential cultures of the three strains grown up to an optical density at 550 nm (OD_{550}) of 0.8 were treated with rifampin (200 μg/ml) and identical cell mass samples were taken after 0, 5, 10, and 15 min of incubation at 70°C. The detection of the *slpA* mRNA was developed by Northern blotting with the specific oligonucleotide O-slpA1. (B) Transcription of the *slpA* gene along the growth of the wild type and the Δ5′UTR mutants. The optical densities at 550 nm of parallel cultures of the HB8 strain (black circles) and its derivatives HB8ΔUTRK1 (white squares) and HBRΔUTR1 (white circles) were monitored along the times indicated. Identical cell mass samples were taken at the times indicated by the black (wild type) and white (mutants) arrows and analyzed for the presence of *slpA* mRNA by Northern blotting.

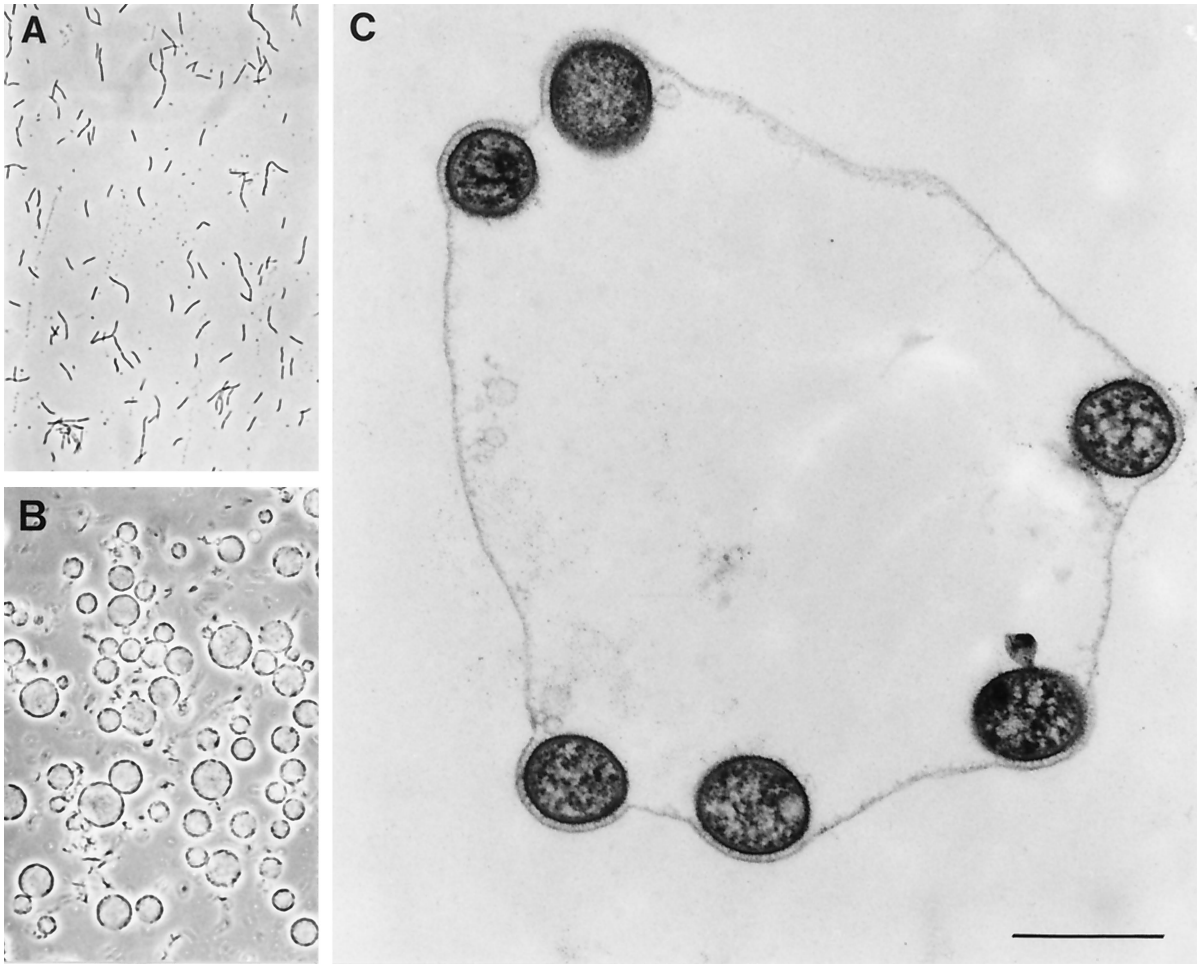

FIG. 3. Effect of the Δ5′UTR mutation on cell morphology. (A and B) Phase-contrast micrographs of overnight cultures of the wild type (A) and the HB8ΔUTR1 mutant (B). (C) Thin section of an overnight culture of the HB8ΔUTR1 mutant showing the intercellular continuity of the external envelope. The bar corresponds to 0.5 μm.

1% (wt/vol) uranyl acetate, and observed under an electron microscope (Phillips EM10).

In Fig. 3C a section of a single MB is shown. As can be seen, an interconnecting envelope binds all the cells within the MB which is continuous with the external envelopes of the cells inside it. These cells had partially incomplete walls, with those areas facing the inside of the MB lacking the outer envelopes (intermediate layer, S-layer, and EDTA-sensitive layer), giving a nude aspect to these regions. Neither cell clusters nor detachment of the outer envelope was observed in wild-type cells.

Conclusions. Deletion of the 5′UTR produces a dramatic reduction in the stability of the *slpA* mRNA and a concomitant increase in the transcription rate from the Δ5′UTR promoter that compensate for the mRNA instability. Thus, the 5′UTR stabilizes the mRNA and represses the transcription from the *slpA* promoter in vivo. In contrast, it does not seem to affect the translation efficiency of the mRNA, as similar amounts of SlpA can be detected in mutants and wild type.

The stabilizing effect of the 5′UTR is probably based on the ability of the leader mRNA to form a highly folded structure (2). Furthermore, this protective effect also could be based on the interaction of the leader mRNA with SlpA (7) in a way similar to the protective role played by the binding of the 30S ribosomal subunit to a Shine-Dalgarno (SD) sequence of the *ompA* leader mRNA (1). On the other hand, the transcription-decreasing effect of the 5′UTR could have a similar structural basis, as the 5′UTR decreases by three- to fourfold the expression of a reporter (β-galactosidase) in *E. coli* (6, 7) in the absence of any other *Thermus* protein.

By contrast, the shutdown of *slpA* transcription when cells stop their growth most probably requires the activity of factors acting in *trans*. The putative contribution of SlrA to this effect is supported by preliminary work showing that transcription of *slpA* remains active for longer periods of time in *slrA* mutants (data not shown). Nevertheless, additional unidentified factors could also play a role in this effect.

The formation of MBs in slow-growing cells is in good agreement with the transcription analysis. Although a detailed analysis of MB formation will be published elsewhere, our current model is based on the oversynthesis of the S-protein with respect to that of the available binding sites on peptidoglycan, with which the S-protein interacts through its amino-terminal SLH motif (11, 12, 13). As a consequence, an excess of S-protein is produced, leading to the formation of blebs that

force other external layers to detach from the peptidoglycan surface, leading to the formation of the MB envelope.

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