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Increasing the stability of sacB transcript improves levansucrase production in *Bacillus subtilis*

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Running title: sacB mRNA stability and protein production

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SUMMARY

Aims: To develop a strategy to increase the stability of transcripts of structural genes expressed under the control of sacR, the leader region of Bacillus subtilis levansucrase gene.

Methods and results: Insertion of Shine Dalgarno like (SD-like) sequences in the 5’ untranslated sacR region controlling the expression of sacB. Depending on the number of stabilizing sequences inserted and the position of these sequences with respect to the translation start codon, it was observed that the mRNA stability and the final protein production could be increased or decreased.

Conclusion: This mRNA stabilization can be used to increase exocellular protein production in the degU32(Hy) mutant.

Significance and impact of the study: This approach can be applied to the expression of heterologous genes of biotechnological interest.

INTRODUCTION

Bacillus subtilis produces and secretes large amounts of various proteins into the culture medium (Priest 1977). For the last two decades numerous attempts have been made to use this bacterium as an efficient host for the expression of proteins (Simonen and Palva 1993). In order to improve the productivity of B. subtilis, efforts have been focused on the development of protease deficient strains (Wong et al. 1995) and identification of efficient regulatory elements at transcription, translation and protein secretion levels (Wu et al. 1991), but the potentiality of transcript stabilization has hardly been investigated. The main factors affecting mRNA stability in bacteria are translation initiation frequency, codon usage and RNA secondary structure (Régnier and Arraiano 2000; Steege 2000). In B. subtilis, ribosome stalling at the 5’ untranslated region (UTR) influences therefore mRNA stability. For example, the stability of aprE, the gene encoding subtilisin which was found to have the highest half-life in B. subtilis (25 min), is due primarily to the gene leader region (Hambraeus et al. 2002). The ribosome binding site (Shine Dalgarno or SD sequence) present in the leader region is considered to be a determinant of stability. The complementarity of the SD sequences at the 3’ OH end of the 16S ribosomal RNA determines the affinity between the ribosomal subunits and the mRNA. The fixation of a ribosomal subunit to the 5’UTR has been reported to be an mRNA stability inducer in B. subtilis (Sharp and Bechhoffer 2003). The presence of SD-like sequences, referred to as stabilizer sequences, in the 5’UTR has been proposed to be one of the causes of the high mRNA stability observed for the cryIIIA gene (Agaisse and Lereclus 1996).

In the present work we aimed to investigate the effect on the stability of the sacB transcript of insertion of SD-like sequences in the sacR region controlling sacB expression. We chose this gene for the following reasons. First, sacB gene coding for extracellular levansucrase is overexpressed under the control of its leader region sacR in a degU32(Hy) strain during the exponential phase of growth (Chambert and Petit-Glatron 1984). The protein was produced in large quantities, even though the half-life of sacB mRNA was only two minutes (Petit-Glatron and Chambert 1992; Pereira et al. 2001). This means that the sacB transcript is characterized by a high lability compared to the average half-life of mRNA in prokaryotes ($t_{1/2} = 6-8$ min) (Hambraeus et al. 2003; Selinger et al. 2003). It can therefore be anticipated that any small change in sacB transcript stability would result in a large measurable change in the production of extracellular protein allowing an accurate measure of the effect of the SD-like sequence insertions. Secondly, a preliminary analysis had been carried out with the levanase structural gene (sacC) expressed under the regulated control of sacR (Pereira et al. 2001). The encouraging results obtained, which involved the role of the sacR UTR region on the stability of the downstream gene transcript, deserved further exploration.
MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study were derivatives of *Bacillus subtilis* QB112 [sacA32, degU32(Hy)] (Chambert and Petit-Glatron 1984) and are listed in Table 1. Cultures were grown aerobically in minimal medium supplemented with 1.5% w/v glucose as a carbon source. Sucrose (50 mmol l⁻¹) was added, when required, into the culture medium at an OD₆₀₀ of 0.2 as described before (Chambert and Petit-Glatron 1984).

Plasmid and strain constructions, *sacR* mutagenesis.

Mutagenesis of *sacR* was done in plasmid pGMC9 (Leloup et al. 1999) using the Quick change site directed mutagenesis kit (Stratagene) with the following oligonucleotides:

- *sacRa fw* and *sacRa rev* (Pereira et al. 2001); *sacRd+a fw* 5'-GGTCTTTTTTATGGTGCGAAAGGAGGAGG -3'; *sacRd+a rev* 5'-CCTCTTTTTTTGTACCTCCTTTGCGGTCTGCTTCTTCCAG -3';
- *sacRe fw* 5'-CTGGAAGAAGCAGACCGCAAAGGAGGTACAT -3';
- *sacRe rev* 5'-CGTTGACGTCTCCTTTTTATGTACCTCCTTTGCGGTCTGCTTCTTCCAG -3'.

The resulting plasmids pGMC15, pGMC16, pGMC17 and pGMC18 contained *sacRa*, *sacRd+a*, *sacRe* and *sacRd+a+e*, respectively (Fig. 1a).

The levansucrase structural gene *sacB* was amplified as described by Daguer et al. (2004). The 1.4 kb DNA fragment obtained by *Aat*II and *Xho*I digestion was purified and ligated into plasmids pGMC9, pGMC15, pGMC16, pGMC17, pGMC18 resulting in plasmids pGMC24, pGMC25, pGMC26, pGMC27, pGMC28. These plasmids were digested with *Bam*HI and *Eco*RV and the different *sacR*-*sacB* fragments were inserted into the integrative plasmid pGMK80 (Daguer et al. 2004) digested with the same enzymes. The plasmids obtained pGMK81, pGMK85, pGMK86, pGMK87 and pGMK88 were used to insert the *sacR*-*sacB* region by double crossing over into the *B. subtilis* chromosome of strain GM96100. Transformants were selected on LB plates for both their resistance to kanamycin (10 µg ml⁻¹) and their sensitivity to spectinomycin (100 µg ml⁻¹) and chloramphenicol (3 µg ml⁻¹). Those exhibiting sucrose inducible expression of levansucrase were chosen and named GM2101, GM2003, GM2004, GM2005 and GM2006.

Isolation and purification of mRNA. RNAs were prepared as described previously by Pereira et al. (2001) from bacteria growing exponentially in the presence or absence of sucrose. Genomic DNA was eliminated by DNase treatment.

Northern blotting. Northern blotting experiments were done essentially as described by Pereira et al. (2001). The *sacR* probe used to determine the half-life and steady state accumulation of mRNA was as described by Pereira et al. (2001). Co-migration with an RNA mass marker (Fermentas) mixture made it possible to estimate the size of the transcripts probed. RNA bands were revealed by phosphor-imaging and quantified with Image Quant software (Molecular Dynamics).

Levansucrase assay

Levansucrase activity was estimated by measuring the initial rate of the fructosyl exchange reaction (Chambert et al. 1974). One unit of enzyme activity corresponds to 2 µg of levansucrase.
RESULTS

Strategy for insertion of Shine Dalgarno like (SD-like) sequences in the sacR region

To select sites of insertion of SD-like sequences (Fig. 1a), we took into account several criteria in order to minimize any modification of the wild-type sequence, to keep space between additional SD-like sequences and to avoid any modification of the ribonucleic antiterminator and terminator sequences, which regulate the expression of sacB gene (Aymerich et al. 1986). We used the same terminology for the various sacR mutants as that adopted in work previously described by Pereira et al. (2001). Prediction of the secondary structures of the mutated sacR sequences showed that the secondary structure of the terminator was preserved in all mutants (Fig. 1b). The transcripts of the sacRd+a and sacRd+a+e regions, however, show a folding conformation that is quite different from the conformations adopted by either the sacR region or the two other mutant sequences sacRa and sacRe (Fig. 1b).

Effect of the mutations on levansucrase production

The quantity of levansucrase released into the culture supernatant of each strain was estimated both by measurements of enzyme activity and by direct quantification of the bands resolved by SDS PAGE (Fig. 2). Production of levansucrase synthesized by strain GM2103 (sacRa-sacB) was 1.5 fold higher than that of strain GM2101 (sacR-sacB). The other mutations decreased the production of levansucrase. The results were the same whatever the growth temperature and inducer concentration used. Moreover, we estimated that the percentage of levansucrase remaining cell associated is lower than 10 % in all mutants indicating that the mutations introduced into the sacR region did not affect the protein secretion mechanism.

Effect of various insertions on the steady state level of sacB transcript

The steady state level of sacB mRNA in each mutant strain grown in the presence of 50 mmol l^-1 sucrose was estimated by Northern blotting using a sacR probe (Pereira et al. 2001) (Fig. 3a). Whereas the sacRa-sacB fusion led to an increase in the cellular level of sacB mRNA by a factor of 1.4 with respect to the wild type, the opposite effect was observed in sacRd+a-sacB and sacRe-sacB mutants. The sacB mRNA level in strain GM2105 (sacRe-sacB) was only one tenth of that in strain GM2101.

Increase in steady state of sacB mRNA results from an increase in sacB mRNA stability

We analyzed the kinetics of sacB mRNA decay in strains GM2101 (sacR-sacB) and GM2103 (sacRa-sacB) by Northern blotting after inhibition of transcription initiation by rifampicin (Fig. 3b). The decay rate evaluated for sacRa-sacB was approximately half that observed for sacR-sacB. Half-lives of the transcripts were 360 s ± 40 and 180 s ± 30, respectively (Fig. 3c). This result indicates that introduction of a second SD-like sequence in the sacR leader region modifies the mRNA stability. The nature of the effect (positive or negative) depends on the position of the additional SD-like sequence.

DISCUSSION

The sacR control region of inducible levansucrase has the important potential to modulate peptide or protein expression in B. subtilis during the exponential phase of growth (Leloup et al. 1997; Pereira et al. 2001; Petit-Glatron and Chambert 1992). The present study shows that introduction of SD-like sequences (stabilizers) in sacR affects the steady-state level of sacB mRNA. The increase observed in one mutant (GM2103) results from the improvement of the sacB transcript stability. The factors that determine mRNA stability in B. subtilis are far from being completely identified (Condon 2003; Hambraeus et al. 2003). Studies on mRNA degradation in E. coli and B. subtilis have shown that the secondary structures in the 5’ leader
of mRNAs are especially important determinants of stability (Hambraeus et al. 2002; Kushner 2002). The presence of a stem-loop structure and the binding of ribosomes can affect the decay rate of transcripts either by directly protecting the mRNA or influencing the structure of the mRNA (Sandler and Weisblum 1989; DiMari and Bechhofer 1993; Agaisse and Lereclus 1996; Sharp and Bechhofer 2003). We postulated that the introduction of a SD-like sequence in the 5’UTR could increase the ribosomal subunit binding rate to the sacR-sacB transcript and affects the kinetics of mRNA degradation.

Steady-state levels result from the synthesis and degradation of RNAs and thus measurement of their degradation rates can be used to determine their rate of synthesis. Comparison of the data obtained from strains GM2101 and GM2103 indicates that SD-like sequence introduction also affects the rate of transcript synthesis since the ratio of the level of the steady state transcript (< 1.5) is lower than that of the degradation rate (> 2). This negative effect could be due to the role played by the sacR transcript in the expression of sacB. The transcript of the leader region is known to fold into a stable terminator which serves as a final transcriptional pause. SacY, the antiterminator protein, prevents termination through its binding to sacR transcript. Therefore any change in the secondary structure of this latter could affect the association between the sacR mRNA and the SacY complex and, consequently, the efficiency of SacY as an antiterminator, and thus the optimization of the transcription machinery.

ACKNOWLEDGEMENTS
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Aymerich, S., Gonzy Tréboul, G. and Steinmetz, M. (1986) 5’ non-coding region sacR is the target of all identified regulation affecting the levansucrase gene in Bacillus subtilis. Journal of Bacteriology 166, 993-998.
Hambraeus, G., Karhumaa, K. and Rutberg, B. (2002) A 5’-stem loop and ribosome binding but not translation are important for the stability of Bacillus subtilis aprE leader mRNA. Microbiology 148, 1795-1803.


Pereira, Y., Chambert, R., Leloup, L., Daguer, J.P. and Petit-Glatron, M.F. (2001) Transcripts of the genes *sacB*, *amyE*, *sacC* and *csn* expressed in *Bacillus subtilis* under the control of the 5’ untranslated *sacR* region display different stabilities that can be modulated. *Microbiology* **147**, 1331-1341.


Figure 1

(a) Schematic representation of the 5’ untranslated region sacR from the start of transcription. The SD-like sequences were introduced into the sacR region as indicated. Variations in the location of the SD-like sequences and the multiplicity of these sequences resulted in the different sacR mutated regions sacRa, sacRe, sacRd+a and sacRd+a+e. The antiterminator RNA sequence (RAT) is indicated by the nucleotides labelled with asterisks and the transcriptional terminator sequence by the nucleotides in boldface. The native Shine-Dalgarno sequence is underlined. The ATG start codon is marked with a star.

(b) The RNA secondary structures of the sacR region of the different mutated sequences which represent the folding of minimum free energies were obtained by computer prediction using Mfold software (Zuker 2003). The star indicates the translation initiation codon, RAT and SD indicate the Ribonucleic AntiTerminator and the native Shine Dalgarno sequence, respectively.

Figure 1  Insertion sites of SD-like sequences in the sacR region
(a) Schematic representation of the 5’ untranslated region sacR from the start of transcription. The SD-like sequences were introduced into the sacR region as indicated. Variations in the location of the SD-like sequences and the multiplicity of these sequences resulted in the different sacR mutated regions sacRa, sacRe, sacRd+a and sacRd+a+e. The antiterminator RNA sequence (RAT) is indicated by the nucleotides labelled with asterisks and the transcriptional terminator sequence by the nucleotides in boldface. The native Shine-Dalgarno sequence is underlined. The ATG start codon is marked with a star.

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Figure 2  Levansucrase production by strain GM2101 and derivatives

The strains were grown in minimal medium supplemented with glucose as carbon source. Sucrose (50 mmol l\(^{-1}\)) was added to the cell suspensions, at an OD\(_{600}\) of 0.2. Samples of the exponentially growing cell suspensions were withdrawn at intervals. Levansucrase was assayed by measuring the initial rate of the fructosyl exchange reaction.

Strains GM2101(●), GM2103(○), GM2104(▲), GM2105(▲), GM2106(●).
Figure 3

(a) **sacB** mRNA steady state and stability analyzed by Northern blotting

Cells of strains GM2101, GM2103, GM2104, GM2105 and GM2106 were grown in minimal medium and levansucrase synthesis was induced with 50 mmol l⁻¹ sucrose at an OD_{600} of 0.5. Samples of the suspensions were withdrawn at OD_{600} = 1.5 and immediately frozen in liquid nitrogen. Total RNA preparations and Northern blotting experiments were carried out as described in Methods. 10 µg of each RNA preparation were loaded and migrated in a 2% agarose gel. Hybridization was carried out with a sacR [³²P]labelled probe as described in Methods. Standardization was achieved by hybridization with an oligonucleotide complementary to the 5S ribosomal RNA. Band intensity was quantified with Image Quant software (Molecular Dynamics). The amount of sacB mRNA is indicated in arbitrary units (AU) using 100 AU of sacB mRNA in strain GM2101 as a reference.

(b) **Kinetic decay of the mRNA transcripts sacR-sacB and sacRa-sacB**

Total RNA was extracted from cultures of strains GM2101 and GM2103 induced by 50 mmol l⁻¹ sucrose. Samples were withdrawn at the times indicated after the addition of rifampicin. Northern blotting analyses were carried out as in (a). Curves were fitted with Sigma Plot software. Strains GM2101 (○), GM2103 (●).
Table 1. Strains and plasmids used in this work

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**Plasmids**

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