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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 OD_{600} 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'-GGTCTTTTTTTATTG TGCGAAAGGAGGTTGCCATCTTGAAAGGAG GAGG-3'; sacRd+a rev 5'-CCTCCT CCT TTCAAGATGGCAACCTCCTTTCGCACAA TAAAAAAAGACC-3'; sacRe fw 5'-CTGGAAGAAGCAGACCGCAAAGGAGGTACAT AAAAAAGGAGACGTCAACG-3'; sacRe rev 5'-CGTTGACGTCTCCTTTTTTATGTACCTCCTTTGCGGTCTGCTTCTT CCAG-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.

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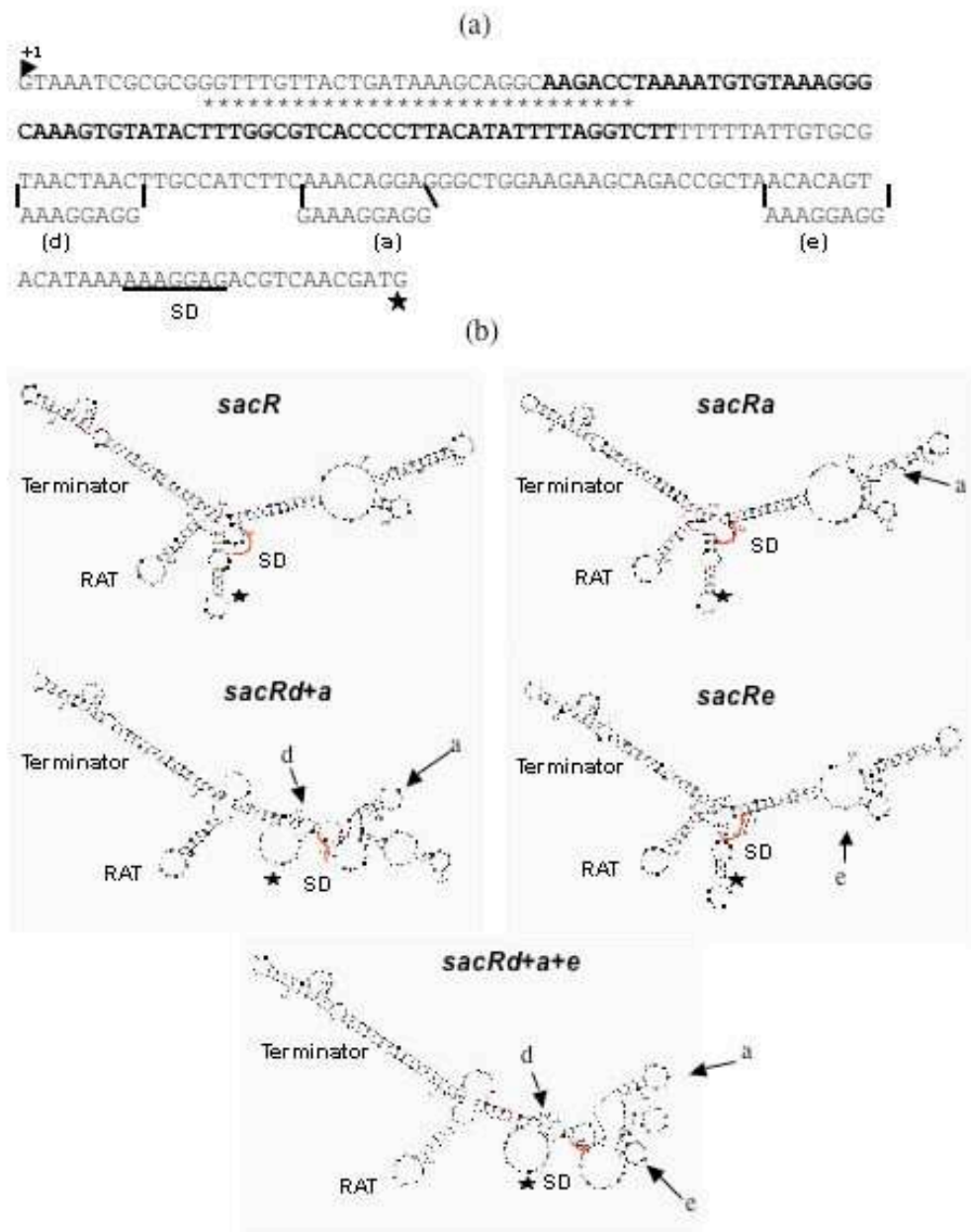
Figure 1

(a)

(b)

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 sequences were introduced into the *sacR* region as indicated. Variations in the location of the SD-like sequences and the mutiplicity 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.
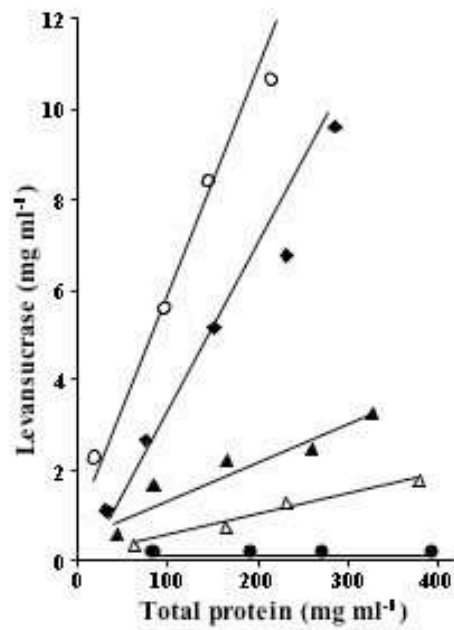
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Figure 2

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.
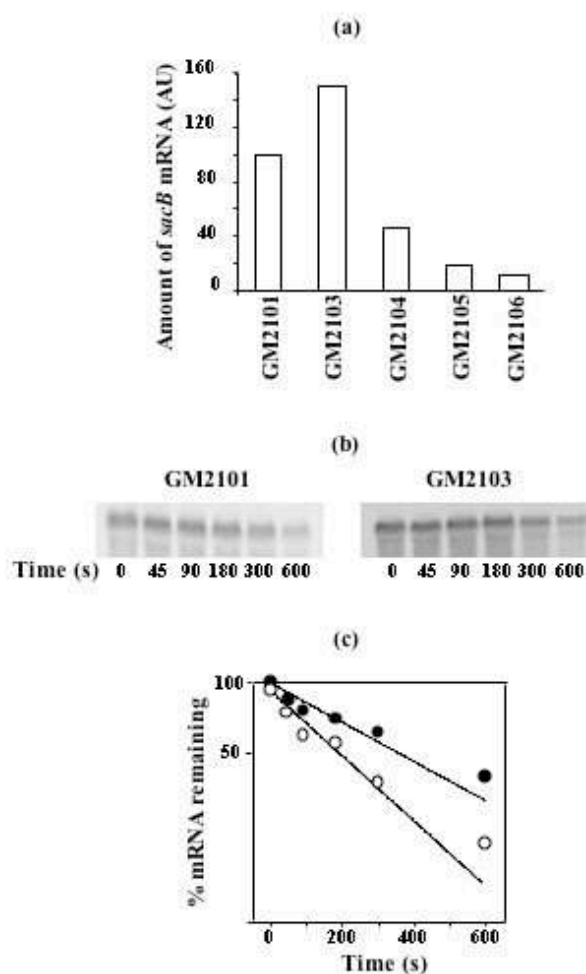Strains GM2101(◆), GM2103(○), GM2104(△), GM2105(▲), GM2106(●).

Figure 3

(a)

(b)

GM2101   GM2103

Time (s) 0 45 90 180 300 600   0 45 90 180 300 600

(c)

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* [^{33}P]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

Strains	Relevant Genotype and Phenotype	Reference or source
QB112	*degU32*(Hy) *sacA321*	Lepesant *et al.* 1974
GM96100	QB112 Δ*sacR-sacB*; Sp^R	Leloup *et al.* 1997
GM2101	GM96100::*sacR-sacB*; Km^R	Daguer *et al.* 2004
GM2103	GM96100::*sacRa-sacB*; Km^R	This work
GM2104	GM96100::*sacRd+a-sacB*; Km^R	This work
GM2105	GM96100::*sacRe-sacB*; Km^R	This work
GM2106	GM96100::*sacRd+a+e-sacB*; Km^R	This work

Plasmids		
pGMK80	pGMKΔ50H1 Km^R, Ap^R, Cm^R	Daguer *et al.* 2004
pGMK81	pGMK80 *sacR-sacB* Km^R, Ap^R, Cm^R	Daguer *et al.* 2004
pGMK85	pGMK80 *sacRa-sacB* Km^R, Ap^R, Cm^R	This work
pGMK86	pGMK80 *sacRd+a-sacB* Km^R, Ap^R, Cm^R	This work
pGMK87	pGMK80 *sacRe-sacB* Km^R, Ap^R, Cm^R	This work
pGMK88	pGMK80 *sacRd+a+e-sacB* Km^R, Ap^R, Cm^R	This work
pGMC9	pCR(+) *sacR* Ap^R	Leloup *et al.* 1999
pGMC24	pGMC9 *sacB* Ap^R	Daguer *et al.* 2004
pGMC15	pCR(+) *sacRa* Ap^R	This work
pGMC16	pCR(+) *sacRd+a* Ap^R	This work
pGMC17	pCR(+) *sacRe* Ap^R	This work
pGMC18	pCR(+) *sacRd+a*+e Ap^R	This work
pGMC25	pCR(+) *sacRa-sacB* Ap^R	This work
pGMC26	pCR (+) *sacRd+a-sacB* Ap^R	This work
pGMC27	pCR(+) *sacRe-sacB* Ap^R	This work
pGMC28	pCR(+) *sacRd+a*+e-*sacB* Ap^R	This work