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

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When *Bacillus subtilis* levanase (SacC), α -amylase (AmyE) and chitosanase (Csn) structural genes were expressed under the regulated control of *sacR*, the inducible levansucrase (SacB) leader region in a *degU32(Hy)* mutant, it was observed that the production yields of the various extracellular proteins were quite different. This is mainly due to differences in the stabilities of their corresponding mRNAs which lead to discrepancies between the steady-state level of mRNA of *sacB* and *csn* on the one hand and *amyE* and *sacC* on the other. In contrast to levansucrase mRNA, the decay curves of α -amylase and levanase mRNAs obtained by Northern blotting analysis did not match the decay curves of their functional mRNA. This suggested that only a part of the population of the *amyE* and *sacC* transcripts was fully translated, while the others were possibly poorly bound to ribosomes and thus were only partially translated or not at all and consequently submitted to rapid endonuclease degradation. This hypothesis was substantiated by the finding that the introduction of a Shine–Dalgarno sequence upstream from the ribosome-binding site in the *sacC* transcript resulted in a fourfold increase in both the half-life of this transcript and the production of levanase. An additional cause of low-level levanase production is the premature release of mRNA by the polymerase. It was attempted to correlate this event with internal secondary structures of *sacC* mRNA.

Keywords: mRNA decay, exocellular proteins, *sacR*, 5' mRNA stabilizer

INTRODUCTION

With its capacity to secrete numerous extracellular enzymes directly into the culture medium (Priest, 1977), *Bacillus subtilis* is a potentially efficient expression host for proteins of interest. Extracellular levansucrase (SacB), expressed under the control of its own leader region (*sacR*), is overproduced in a *degU32(Hy)* mutant during the exponential phase of growth (Chambert & Petit-Glatron, 1984) and represents 5 % of total proteins

under conditions of full induction by sucrose. This *sacR* leader region has therefore been widely used for the inducible secretion of heterologous proteins during the exponential phase of growth (Joliff *et al.*, 1989; Dion *et al.*, 1989; Wong, 1989; Petit *et al.*, 1990). Surprisingly, the extracellular production of these proteins, including those originating from other bacilli (Joyet *et al.*, 1986), is within one order of magnitude lower than that of levansucrase. For a long time this low level of production was considered to be due to the susceptibility of foreign proteins to extracellular proteases. Recently, two native secreted proteins, α -amylase (AmyE) and levanase (SacC), which are normally expressed at a low level during the stationary phase of growth under the control of their own promoter, were expressed in *B. subtilis*

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Abbreviation: SD, Shine–Dalgarno.

under the control of *sacR* (Leloup *et al.*, 1997, 1999). In the *B. subtilis* 168 wild-type strain, *amyE* is controlled by its promoter *amyR1* (Yamaguchi *et al.*, 1974) and *sacC* is the fifth gene of an operon in which the first four genes (*levD*, *levE*, *levF* and *levG*) encode polypeptides that constitute a minor fructose phosphotransferase system involved in the regulation of levanase expression (Martin-Verstraete *et al.*, 1990). The structural parts of *amyE* and *sacC* (including the sequence of their own signal peptide) were fused to *sacR* and the gene fusions were introduced into the chromosome of a *degU32*(Hy) mutant. These strains produced α -amylase and levanase in the culture medium during the exponential phase of growth in the presence of sucrose as an inducer. Even so, under these conditions the yield was still much lower than that of levansucrase (Leloup *et al.*, 1997, 1999). Pulse-chase experiments indicated that there were no specific bottlenecks in the secretion pathway of α -amylase and levanase to account for such differences, since the precursor form of each protein is efficiently processed (Leloup *et al.*, 1997, 1999). Therefore, it was postulated that the mRNA steady-state level controlled by mRNA decay and/or translation mechanisms is adapted to a particular growth phase (Shields & Sharp, 1987; Saier, 1995).

To test this hypothesis we assessed the transcriptional efficiency of the gene fusions and the stability of the resulting transcripts. This comparative study was enlarged to include chitosanase, a native extracellular protein that is produced at a low level during the exponential growth phase in *B. subtilis* 168 (Y. Pereira and others, unpublished results). The structural gene *csn* was expressed in a *degU32*(Hy) mutant under the control of the inducible levansucrase leader region *sacR*. Thus the transcripts of the four different structural genes shared a similar 5' untranslated region encompassing the translation initiation signals and possessed 3' ρ -independent terminators of very similar calculated free energies. In contrast, the original transcriptional context of these genes is varied, since they are either mono or polycistronic (*sacC*), lie on either the leading (*sacB* and *amyE*) or the lagging DNA strand (*csn* and *sacC*) and are expressed during either the exponential (*sacB* and *csn*) or stationary (*amyE* and *sacC*) growth phase.

Little is known about the mechanism of mRNA degradation in *B. subtilis* unlike that of *Escherichia coli* (Coburn & Mackie, 1999; Régner & Arraiano, 2000; Steege, 2000). Three RNases involved in mRNA processing in *B. subtilis* have been identified: an endonuclease, RNase III (Wang & Bechhofer, 1997), and two 3'-5' exonucleases, polynucleotide phosphorylase and the *yvaJ* gene product (Wang & Bechhofer, 1996; Bechhofer & Wang, 1998; Oussenko & Bechhofer, 2000). It is generally considered that most mRNAs are protected against 3' exonuclease activities by a stable secondary structure at their 3' end independently from the nucleotide sequence (Higgins *et al.*, 1993), and that degradation is initiated by upstream endonucleolytic cleavage (Bouvet & Belasco, 1992) that depends mainly on 5' binding and sliding of endonucleases, although no

homologue of the *E. coli* RNase E has yet been identified in *B. subtilis* (Kunst *et al.*, 1997; Condon *et al.*, 1997; Kaberdin *et al.*, 1998). Furthermore, transcripts of Gram-positive bacteria have been shown to contain 5' motifs that confer stability on heterologous downstream mRNAs (Bechhofer, 1993). Such motifs are the 5' mRNA stabilizer of the *erm* genes of *B. subtilis* (Bechhofer & Dubnau, 1987; Bechhofer & Zen, 1989), the 5' poly-purine sequence of the *B. subtilis* SP82 phage (Hue *et al.*, 1995) and the Shine-Dalgarno (SD)-like sequence of the *cryIIIA* mRNA of *Bacillus thuringiensis* (Agaisse & Lereclus, 1996) which form stable complexes with ribosomes in *B. subtilis*. To test this potentiality further, we introduced a short sequence complementary to the end of the 16S rRNA in different places in the *sacR* region of the *sacC* transcript.

We show in this study that differences in the stabilities of the respective mRNAs may account for the differences in the production yield of the four native exoproteins. Moreover, the presence of a 5' mRNA stabilizer upstream from the RBS of *sacC* can lead to an increase in both the steady-state level and the stability of the mRNA and likewise to an increase in the amount of levanase released into the culture supernatant.

METHODS

Bacterial strains and media. The strains and plasmids used in this work are listed in Table 1. All were derivatives of the *degU32*(Hy) *Bacillus subtilis* mutant. Strains were grown at 37 °C in minimal medium (Chambert & Petit-Glatron, 1984) supplemented with 1% (w/v) glucose and 0.5 mM calcium chloride. Strain GM9804 contained a *sacRcsn* fusion and strains GM96202, GM96203 and GM96204 contained fusions of *sacR* mutants with *sacC* as described below.

Construction of *sacRcsn* fusion and expression of chitosanase. The *B. subtilis* chitosanase structural gene was identified by analysis of the complete genome sequence (Kunst *et al.*, 1997). An ORF encoding chitosanase (28 kDa) was identified in the 233 ° region of the *B. subtilis* genome. This enzyme shares 36.4% identity with the chitosanase product of the *Streptomyces* sp. *csn* gene (Masson *et al.*, 1994; Parro *et al.*, 1997) and presents the same activity as that assayed using glycol-chitosan as substrate (Tominaga & Tsujisaka, 1975). The *B. subtilis* structural gene *csn* was amplified by PCR with primers *csn1* and *csn2*, including restriction sites, as indicated in Table 2, from the chromosomal DNA of strain QB112 isolated as described previously (Leloup *et al.*, 1997). The amplified blunt-ended fragment was inserted into the pCR(+) vector at the *SrfI* site, after appropriate treatment according to the supplier's recommendations (Stratagene). The *csn* fragment was isolated after digestion with *SphI* and *XhoI* and ligated into the pCR(+) *sacR* vector pGMC8 (Leloup *et al.*, 1997) digested with the same enzymes. In pGMC8, which was used previously to construct the fusion *sacRamyE* (Leloup *et al.*, 1997), the *SphI* site in *sacR* fragment was created by substituting the original RBS sequence 5'-AAAGGAGACA-TGAACG-3' with the following sequence: 5'-AAAGGAGG-CATGCAACG-3'. The resulting plasmid pGMC12 was used to transform strain *E. coli* XL-1 Blue. One transformant was selected and the complete sequence of the gene fusion carried by the recombinant plasmid was controlled by sequencing of the double-stranded recombinant DNA with the appropriate

Table 1. Strains and plasmids

Strain/plasmid	Relevant genotype and phenotype	Source or reference
Strains		
QB112	<i>degU32(Hy) sacA321</i>	Lepesant <i>et al.</i> (1976)
GM96100	<i>degU32(Hy) sacA321 ΔsacRsacB; Sp^R</i>	Leloup <i>et al.</i> (1997)
GM96101	<i>degU32(Hy) sacA321 ΔsacRsacB sacRamyE; Km^R Sp^R Cm^R</i>	Leloup <i>et al.</i> (1997)
GM96201	<i>degU32(Hy) sacA321 ΔsacRsacB sacRsacC; Km^R Sp^R Cm^R</i>	Leloup <i>et al.</i> (1999)
GM9804	<i>degU32(Hy) sacA321 ΔsacRsacB sacRcsn; Km^R</i>	This work
GM96202	<i>degU32(Hy) sacA321 ΔsacRsacB sacRasacC; Km^R Sp^R Cm^R</i>	This work
GM96203	<i>degU32(Hy) sacA321 ΔsacRsacB sacRbsacC; Km^R Sp^R Cm^R</i>	This work
GM96204	<i>degU32(Hy) sacA321 ΔsacRsacB sacRcsacC; Km^R Sp^R Cm^R</i>	This work
Plasmids		
pLS50	<i>B. subtilis sacRsacB</i> region inserted into pJH101; Cm ^R	Steinmetz <i>et al.</i> (1985)
pGMK50	pLS50 derivative; Km ^R Cm ^R Ap ^R	Chambert <i>et al.</i> (1992)
pGMK70	pLS50 derivative; Km ^R Cm ^R Ap ^R	This work
pGMK72	pGMK70 <i>sacRcsn</i> ; Km ^R Cm ^R Ap ^R	This work
pCR(+)	Ap ^R	Stratagene
pGMC8	pCR(+) <i>sacR</i> ; Ap ^R	Leloup <i>et al.</i> (1997)
pGMC9	pCR(+) <i>sacR</i> ; Ap ^R	Leloup <i>et al.</i> (1999)
pGMC12	pCR(+) <i>sacRcsn</i> ; Ap ^R	This work
pGMC15	pCR(+) <i>sacRasacC</i> ; Ap ^R	This work
pGMC16	pCR(+) <i>sacRbsacC</i> ; Ap ^R	This work
pGMC17	pCR(+) <i>sacRcsacC</i> ; Ap ^R	This work

Table 2. Oligonucleotides used in this study

Oligonucleotide (5'–3')*	Restriction site at the 5' end
<p style="text-align: center;"><i>Bam</i>HI <i>Sph</i>I <i>Xho</i>I <i>Sma</i>I</p> <p>MCS in pGMK70: CCGGATATCGGGGATCCCAAGCTT<u>G</u>CATGCGCTCGAGTCGACCCCGGG</p> <p>GATCTATTGGCATTCTTTCTTTGCTAGCAATATTGC</p> <p>cat1: ACAGCTGCCTTCTTCAACTAACGGGCAG</p> <p>cat2: TGCATGCATGTGTTACAGTAATATTGAC</p> <p>csn1: GGAGGCATGCAACGATGAAAATCAGTATGC</p> <p>csn2: GGGCTCGAGGCCTTCTTGACAGATCCATTG</p> <p>5S rRNA probe: ACTACCATCGGCGCTGAAGA</p> <p>SacRa: CTAACCTTGCCATCTTGAAAGGAGGAGGGCTGGAAGAAGC</p> <p>Complementary sacRa: GCTCTTCCAGCCCTCCTCTTCAAGATGGCAAGTTAG</p> <p>SacRb: GTGCGTAACTAACTTGCAGAAAGGAGGCATCTTCAAACAGGAGG-3'</p> <p>Complementary sacRb: CCTCCTGTTTGAAGATGCCTCCTTTCTGCAAGTTAGTTACGCAC</p> <p>SacRc: GGTGCAAAAGTAAATAGAAAGGAGGCGCGCGGTTTGTACTG</p> <p>Complementary sacRc: CAGTAACAAACCGCGCGCTCCTTTCTATTTACTTTTTTCGACC</p> <p>lev1: CATCCGGATACAGCGCAACAGGAAGGTG</p> <p>lev2: GGCAATACTTTGCACTTGATGGCCTTCCCG</p>	<p><i>Eco</i>RV</p> <p><i>Ssp</i>I</p> <p><i>Pvu</i>II</p> <p><i>Nsi</i>I</p> <p><i>Sph</i>I</p> <p><i>Stu</i>I</p>

* Letters in bold refer to restriction sites at the 5' end or to *sacR* modifications.

synthetic oligonucleotides. The *Bam*HI–*Stu*I fragment was purified and ligated into pGMK70 digested with *Bam*HI/*Sma*I, resulting in pGMK72 (Fig. 1). Strain GM9804, obtained by transformation of *B. subtilis* GM96100 with pGMK72, over-produces chitosanase in the supernatant of exponentially growing cells in the presence of sucrose. The differential rate

of chitosanase production was estimated with reference to protein markers by densitometric analysis of a Coomassie-blue-stained SDS-polyacrylamide gel using NIH Image software.

Isolation of total cellular RNA. Bacteria (5 ml) from an

Fig. 1. Construction of the integrative plasmid pGMK72. The region from pBR322 is indicated as a black box and contains the ColE1 origin of replication in *E. coli* and *bla* encoding resistance to ampicillin (Ap). Fragments I and II that flank the *sacR**sacB* region in the *B. subtilis* chromosome were introduced on either side of a synthetic oligonucleotide containing cloning sites (Table 2). They correspond to an *NsiI*–*SspI* nucleotide sequence of 673 bp and a *NaeI*–*HindIII* sequence of 428 bp, and to nucleotides 3533914–3534587 and 3536948–3537376 of the *B. subtilis* genome sequence, respectively. Km indicates the gene encoding kanamycin resistance from pUB110, which was purified from pBest502 (Petit-Glatron & Chambert, 1992); Cm indicates the *cat* gene from pC194 (Horionouchi & Weisblum, 1982), which encodes resistance to chloramphenicol and was cloned by PCR with primers cat1 and cat2 (Table 2).

exponentially growing culture were collected at an OD₆₀₀ of 1.5 and immediately frozen with liquid nitrogen. Total RNA was isolated essentially as described by Putzer *et al.* (1992) by vigorous vortexing of the cells in an ice-cold mixture containing 5 g glass beads (100 µm diam.; Braunschweig), 5 ml phenol, 1 ml chloroform, 0.2% (w/v) bentone (Bentone MA; Rheox) and 0.2% (w/v) SDS. After phenol and chloroform extractions of the aqueous phase, followed by a 2-propanol precipitation and 70% (v/v) ethanol washing of the pellet, RNA was resuspended in an appropriate volume of diethylpyrocarbonate-treated water.

mRNA half-life measurements. Rifampicin (100 µg ml⁻¹ final concentration) was added to exponentially growing cultures at an OD₆₀₀ of 1.5. Aliquots of 5 ml culture suspension were collected at intervals and immediately frozen in liquid nitrogen. RNAs were prepared as described above and mRNA half-lives were determined after Northern blotting with labelled probes as follows. RNA bands were revealed by phosphor-imaging and relative amounts of mRNA at each time quantified using ImageQuant software (Molecular Dynamics). The kinetic data of mRNA decay were analysed by

Fig. 2. Free energies (ΔG_0) of 3' end ρ -independent terminators of the *amyE*, *sacC*, *csn* and *sacB* transcripts, and probes used in Northern blotting experiments. The fragments corresponding to specific probes (thick black lines) used in the Northern blotting experiments and their lengths are indicated. Nucleotides are numbered from the transcription start (+1). ΔG_0 values were estimated using the MFOLD program (Zuker *et al.*, 1999).

non-linear least squares fitting to the sum of exponentials (SigmaPlot curve fitter program):

$$(mRNA)_t = \sum_{i=1}^n (mRNA^0)_i \cdot e^{-\lambda_i t}$$

where $(mRNA^0)_i$ is the initial amount of species i , and λ_i is the apparent rate constant of the species i decrease. Two models were proposed, a monophasic decay ($i = 1$) or a biphasic decay ($i = 2$). The model which fitted the data with the minimum deviation in each case was retained as being the more valid.

Northern blotting. Total RNA samples (5 or 10 µg) were separated by electrophoresis on agarose gels under the conditions described by Ausubel *et al.* (1994) and then transferred onto a positively charged polyamide membrane (Nytran +; Schleicher & Schuell) in 20 × SSC using a vacuum blotter (Hybaid). Hybridization with the probes described in Fig. 2 was carried out in a solution containing 5 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7), 5 × Denhardt's solution, denatured salmon sperm DNA (100 µg ml⁻¹ final concentration) and 1% (w/v) SDS in 50% (v/v) formamide at 42 °C, overnight. Labelling of the probes was performed either by random priming (Megaprime; Amersham) and [α -³²P]dATP or with T4 polynucleotide kinase and [γ -³²P]dATP. In addition to the initial quantification of the total RNA by spectrophotometric measurements before electrophoresis on agarose gel, 5S rRNA, used as an internal control for standardization of the RNA samples, was probed with a 5' end-labelled oligonucleotide (Table 2), which anneals to the 5S rRNA between nucleotides 59 and 78. Co-migration with a molecular mass marker mixture (9488–363 nt RNA molecular mass markers; USB) made it possible to estimate the length of the transcripts. RNA bands were revealed by phosphor-imaging and quantified with ImageQuant software.

α -Amylase and levanase activity assays. α -Amylase activity was assayed at 37 °C using *p*-nitrophenylmaltotriose as a substrate (bioMérieux) as described previously (Leloup *et al.*, 1997). Levanase activity was assayed using uniformly labelled [¹⁴C]levan as substrate (Leloup *et al.*, 1999).

Fig. 3. Schematic representation of the 5' untranslated region, *sacR*, from the start of transcription. Mutations in *sacR*, *sacRa*, *sacRb* and *sacRc*, are indicated. The nucleotides labelled with asterisks represent the antiterminator RNA sequence and the inverted arrows represent the transcription terminator. The putative SD sequence is underlined.

Fig. 4. Northern blotting analysis of total RNA extracted from strains producing α -amylase (GM96101), levanase (GM96201), chitosanase (GM9804) and levansucrase (QB112) under the control of *sacR*. Cells were grown in minimal medium in the presence (+) or absence (–) of sucrose. Samples of the culture were withdrawn at an OD₆₀₀ of 1.5, immediately frozen in liquid nitrogen and then treated as described in Methods. Ten micrograms of each RNA preparation was analysed by Northern blotting. Hybridization was done with a ³³P-labelled *sacR*-specific probe. Migration of the 23S (2928 nt) and 16S (1553 nt) rRNAs is indicated on the right. The short transcript of *sacC* is labelled with an asterisk.

***sacR*-directed mutagenesis.** Plasmid pGMC9, containing the *sacR* sequence with a minor modification (Leloup *et al.*, 1999), was used to introduce a 5' mRNA stabilizer sequence by the

Quick change site-directed mutagenesis method (Stratagene) with complementary oligonucleotides (Table 2) to create, by substitution (*sacRa*) or insertion (*sacRb* and *sacRc*), a sequence 5'-GAAAGGAGG-3' at various positions as shown in Fig. 3. This gave pGMC15, pGMC16 and pGMC17 containing the *sacRa*, *sacRb* and *sacRc* mutant fragments, respectively. The structural gene of *sacC* was fused to the different *sacR* fragments. The resulting *sacRscC* fusions were controlled by sequencing, ligated into pGMK50 and used to transform strain GM96100 as described previously (Leloup *et al.*, 1997), giving strains GM96202, GM96203 and GM96204, respectively.

RESULTS

The production of SacB, SacC, AmyE and Csn, when their corresponding genes are expressed under the control of *sacR*, correlates with the steady-state level of their mRNAs

Strains GM96101 and GM96201, which express *sacRamYE* and *sacRscC* gene fusions, were constructed as described previously (Leloup *et al.*, 1997, 1999). The construction of strain GM9804 expressing *sacRcsn* is described in Methods. Northern analyses of the cellular RNAs of these strains growing exponentially in the presence or absence of sucrose were performed in parallel to that of strain QB112 producing levansucrase (SacB) using either the 5' *sacR* probe for hybridization in the 5' region of the transcripts or internal probes of the respective genes (Fig. 2). Transcripts of each gene were synthesized when the strains were cultivated in the presence of sucrose (Fig. 4). These transcripts were of the expected lengths (about 2200, 2400, 1100 and

Table 3. Production of *B. subtilis* exoproteins and steady-state levels of their mRNA

Protein	μg (mg total protein) ⁻¹	pmol (mg total protein) ⁻¹	Ratio to levansucrase	mRNA steady-state level ratio to levansucrase mRNA
Levansucrase	52.5	1.05	1.00	1.00
α -Amylase	31.2	0.45	0.43	0.46
Levanase	6.9	0.09	0.09	0.13
Chitosanase	21.2	0.77	0.74	0.98

Fig. 5. Kinetic decay of the mRNAs transcribed from *amyE*, *sacC*, *csn* and *sacB*. Cells were grown in the presence of 60 mM sucrose. Total RNAs were prepared from samples withdrawn at the indicated times starting 45 s after the addition of the transcription inhibitor. The gels were loaded with 10 µg RNA per lane. Northern blotting analysis was performed with gene-specific probes and/or the *sacR* probe. A representative Northern blotting experiment is shown here with the curve of mRNA decay obtained from SigmaPlot curve fitting treatment as described in Methods.

1650 nt, for *amyE*, *sacC*, *csn* and *sacB*, respectively), in agreement with the previously determined site of transcription initiation in the *sacR* leader sequence (Shimotsu & Henner, 1986) and generated by transcriptional antitermination at the terminator structure present in *sacR* (Crutz *et al.*, 1990). A short additional transcript which hybridized only with the 5' *sacR* probe was observed in the case of *sacC* (Fig. 4); it might have been the result of either early transcription termination or degradation. The size of this 3' truncated transcript was evaluated to be about 550 nt by electrophoresis on an acrylamide gel (Ausubel *et al.*, 1994) and by Northern

blotting using the *sacR* probe (not shown). This point will be discussed later.

Quantification of the labelled bands indicated that the steady-state levels of *sacB* and *csn* transcripts were similar, whereas *amyE* and *sacC* (full-length) levels were 46 and 13 %, respectively, of that of *sacB*. The mRNA levels are, thus, well correlated to the extracellular production of the corresponding proteins (Table 3). The reasons for such a difference in the mRNA steady-state levels could be attributed to differences in either the transcriptional efficiencies of the various genes or their mRNA stability. We therefore analysed the kinetics of decay of these messengers.

Comparison of the kinetics of mRNA decay from Northern blotting analysis with the kinetics of functional mRNA decay

Stability analysed by Northern blotting. The kinetics of mRNA decay were studied under the same conditions for the four RNA preparations after inhibition of transcription initiation. RNA preparations were analysed by Northern blotting and hybridized with 3'-specific probes and/or the 5' *sacR* probe (Fig. 5). The data obtained from the quantification of the bands on the Northern blot from three independent experiments were analysed as mono or biphasic exponential decay with the SigmaPlot curve fitter program. The results (Table 4) show that levansucrase and chitosanase mRNA decays are monophasic events displaying the same time constant. The decays of full-length and short transcripts of *sacC* appear to be monophasic; both transcripts are more unstable than *sacB* and *csn* mRNAs.

The decay of *amyE* mRNA best fitted a biphasic event, suggesting that two mRNA populations, with quite different stabilities, are present in approximately equivalent proportions under steady-state conditions (Fig. 5). It is worthwhile noting that the time constant of the decay of the more stable of the two is close to those of *sacB* and *csn* transcripts.

Stability of functional mRNAs. The functional mRNA decay was evaluated by quantifying the increase in the amount of enzyme synthesized after inhibition of transcription initiation. The half-life of levansucrase functional mRNA determined previously for the same strain under the same growth conditions (Chambert & Petit-Glatron, 1984) was evaluated at 100 ± 7 s. A similar approach was experimentally suitable for AmyE and SacC, but not for Csn, due to the poor sensitivity of the assay method of this enzyme (Tominaga & Tsujisaka, 1975). Our results (Fig. 6a, b) show that the functional mRNA decay of *sacRamyE* and *sacRsacC* constructs fitted with monophasic events. Their half-lives were estimated to be 103 ± 21 and 129 ± 18 s, respectively. For α -amylase, the value of the functional half-life (103 s) was close to that of the half-life of the more stable transcript species (Fig. 5) determined by direct measurement by Northern blotting. For levanase, the value was much higher than that of the full-length mRNA.

Table 4. Half-lives of *sacRamYE*, *sacRsacC*, *sacRcsn* and *sacRsacB* mRNAs

mRNA	Single exponential		Double exponential			
			Fast phase		Slow phase	
	Time constant $10^2 \times k$ (s ⁻¹)	Half-life (s)	Time constant $10^2 \times k_1$ (s ⁻¹)	Half-life (s)	Time constant $10^2 \times k_2$ (s ⁻¹)	Half-life (s)
<i>sacRamYE</i>	—	—	3.57 ± 1.29	19 ± 7	0.56 ± 0.15	124 ± 34
<i>sacRsacC</i> full-length	1.30 ± 0.04	53 ± 2	—	—	—	—
<i>sacRsacC</i> short transcript	1.83 ± 0.08	38 ± 2	—	—	—	—
<i>sacRcsn</i>	0.46 ± 0.04	151 ± 13	—	—	—	—
<i>sacRsacB</i>	0.5 ± 0.11	139 ± 31	—	—	—	—

Stabilization of the *sacRsacC* transcript

The results obtained from the analysis of mRNA stability indicated that a similar 5' untranslated region is not sufficient to confer identical stability on the downstream transcript. Furthermore, comparison of the apparent biphasic decay of total *amyE* mRNA with the monophasic decay of the functional transcript suggested that a significant proportion of the *amyE* transcript is not ribosome-bound and is consequently subjected to rapid degradation. This part might be so great in the case of the *sacC* (full-length) transcript that mRNA decay appears as a monophasic event. One can thus ask whether an increase in the potential of mRNA to bind to ribosomes in turn increases the mRNA stability. We investigated whether insertion of a 5' mRNA stabilizer has such an effect, as previously suggested by Bechhofer & Dubnau (1987), with the *sacRsacC* fusion for two reasons: first, the mRNA has the lowest half-life of all the transcripts studied here, making a potential detection of changes in the stability easier and, second, it should make it possible to determine the status of the short *sacC* transcript. We therefore introduced as a 5' mRNA stabilizer, a sequence analogous to the SD element whose bases pair with the 3' end of the 16S rRNA subunit. Three mutants were constructed as described in Methods (Fig. 3). The mutant strains were grown in parallel with the reference strain (GM96201) under the same conditions as before. Total cellular RNA was extracted at various times after inhibition of transcription initiation and probed with the 5' *sacR* fragment. Two discrete bands were observed in the Northern blot of each mutant strain (Fig. 7), corresponding to the full-length and the short transcripts also found in the reference strain (about 2200 and 550 nt). The decays of the two mRNA species both follow monophasic kinetics. The effects of the 5' mRNA stabilizer on the longevity of the *sacRsacC* transcripts are summarized in Table 5. A significant increase in the half-life of the *sacC* full-length transcript was observed in all mutant strains. The highest values were obtained in strains GM96202 and GM96203 in which the additional SD sequences are close (34 and 52 nt, respectively) to the RBS. We found that the steady-state levels

of the *sacRasacC*, *sacRhsacC* and *sacRcsacC* mRNAs, evaluated under the same conditions as *sacRsacC* mRNA taken as reference, are in direct correlation with their decays (Table 5). The production of levansucrase in the supernatants of the three mutant strains was increased fourfold in strain GM96202 only, while it remained unchanged in GM96203 and decreased by 45% in GM96204. The relative amount of the short transcript was approximately the same in the three mutants and the reference strain. Oligonucleotide probes lev1 and lev2 (Table 2) were synthesized to determine the location of the 3' end of the short transcript. Probe lev1, complementary to the nucleotides 464–492, hybridized with the four short transcripts, whereas lev2, complementary to nucleotides 596–625, did not (not shown).

DISCUSSION

Levansucrase, α -amylase, levansucrase and chitosanase, four native extracellular proteins of *B. subtilis*, expressed under the control of the levansucrase 5' untranslated leader region, *sacR*, are produced at very different levels in the culture supernatants.

Measurements of the steady-state level and stability of the mRNAs of the four proteins also indicated differences. Whether these differences could be caused by the minor modifications introduced in the RBS spacer regions of *sacR* in the various constructs should be discussed. First, the *sacRamYE* transcript has a shorter half-life than *sacRcsn* and the production of α -amylase is lower than that of chitosanase, although the two transcripts possess identical spacer regions (length and base sequence). Second, in contrast, *sacRsacB* and *sacRcsn* transcripts, which show two differences (one substitution and one insertion) in their spacer region, present no significant differences in their stability, and the production of chitosanase and levansucrase is similar under the same conditions. Finally, during the revision process of the manuscript, we constructed a second *sacRsacB* fusion, based on the *sacR* fragment of plasmid pGMC9 (Leloup *et al.*, 1999), which was also used to construct the *sacRsacC* gene fusion. We observed an identical production of levansucrase by the strain

Fig. 6. Determination of the functional decay of *amyE* (a) and *sacC* (b) mRNAs. The functional half-lives of *amyE* and *sacC* mRNA were determined by quantifying the amount of α -amylase and levanase produced at intervals after inhibition of transcription. Cells of strains GM96101 or GM96201 were induced at an OD_{600} of 2.5 with 60 mM sucrose. After 20 min induction, rifampicin was added at $100 \mu\text{g ml}^{-1}$ final concentration. Aliquots of cell suspension were removed at time intervals and immediately centrifuged at 2°C in the presence of chloramphenicol ($100 \mu\text{g ml}^{-1}$ final concentration). α -Amylase or levanase was subsequently assayed in the supernatants. The mean value and the standard deviation of four independent assays of enzyme activity for each sample are plotted as a function of time after addition of rifampicin.

harbouring this fusion, despite the two substitutions in the spacer region (data not shown). From this set of results, we concluded that the modifications introduced in the RBS spacer regions of the various constructs could not account for the discrepancies observed in the stability and in the translational efficiency of their respective mRNAs. We can now discuss other hypotheses.

In prokaryotic cells various factors are known to affect mRNA stability and therefore the steady-state level of the transcript. These factors include both gene-specific characteristics and bulk cellular conditions (Carrier &

Kiesling, 1997), and can be divided into several categories which we will examine below.

The effects of protein synthesis on mRNA stability include translational initiation and elongation rate as well as codon usage. It has been noted that the expression of proteins can be controlled by codon usage according to the cellular levels of tRNA, which fluctuate with growth conditions and growth phase (Saier, 1995; Shields & Sharp, 1987; Karlin & Mrazek, 2000). However, the different gene fusions constructed were all expressed under the same growth conditions and during the same growth phase. Moreover, comparison of codon usage in *amyE*, *sacC* and *csn* showed that it is very similar for all three, but differs greatly from that of *sacB* (Nitschké *et al.*, 1998). Thus, a global effect of codon usage is highly improbable, although one should not rule out the possibility of a local effect on the rate of elongation which leaves sites unprotected from endonucleolytic attack.

Moreover, the 5' untranslated region contains a hairpin (ribonucleic antiterminator sequence) involved in the antitermination mechanism (Crutz *et al.*, 1990; Aymerich & Steinmetz, 1992) which regulates the transcription of genes under the control of *sacR* and can protect them, at least partially, from 5' endonuclease attack (Emory *et al.*, 1992; Bechhofer, 1993). The free energies of stabilization of the four terminators at the 3' end are all very similar, therefore excluding the hypothesis that the differential stability is caused by differences in hairpins, which are supposed to prevent exonucleases from degrading the coding sequence of the mRNA (Higgins *et al.*, 1993). Finally, the reasons for the low stability of *amyE* and *sacC* transcripts could lie in the translation mechanism adapted to a particular growth phase (Saier, 1995), hence the features of the translation machinery of the exponential phase did not fit well with these transcripts. The double exponential decay of *amyE* mRNA in addition to the monophasic decay of the corresponding functional mRNA suggests that this mRNA exists in two different states in the cells: one state with a very short half-life, probably either unprotected or only poorly protected by ribosomes, and therefore highly sensitive to endonucleolytic attack, and the other ribosome-bound with a half-life within the same range as that of *sacB* and *csn* transcripts. A similar hypothesis concerning the coexistence in the cell of functional and inactivated mRNA was proposed by Petersen (1993).

This hypothesis was supported by the effects of a 5' mRNA stabilizer on the stability of *sacC* mRNA. The effects strongly depend on the distance between the sequence and the RBS. An increase in levanase production was observed mainly in strain GM96202 (*sacRasacC* fusion) where the SD sequence was created by punctual mutations and was located at a distance of 34 nt from the RBS without any ATG codon between that could modify the initiation of translation. Such an increase unambiguously correlated with an increase in the steady-state level of mRNA resulting from an

Fig. 7. Kinetic decay of the mRNAs transcribed from the gene fusions of *sacRa*, *sacRb*, *sacRc* with *sacC*. Cells were grown as described in the legend to Fig. 5. Total RNAs were prepared from samples withdrawn from exponentially growing culture at the indicated times starting 45 s after the addition of the transcription inhibitor. The gels were loaded with 10 µg RNA per lane. Northern blotting analysis was performed with the *sacR* probe from 0 to 180 s (a). Quantification of the bands corresponding to the full-length transcripts was carried out as described in Methods. The decays of the full-length transcripts are represented in (b): ◇, *sacRasacC*; △, *sacRbsacC*; ○, *sacRcsacC*; □, *sacRsacC*.

Table 5. Half-lives of *sacRsacC* mutant mRNAs

Strain	Single exponential				Steady-state levels (relative ratio)	Secreted levanase production	
	Full length		Short mRNA			$\mu\text{g (OD}_{600}\text{ unit)}^{-1}\text{ ml}^{-1}$	Relative ratio
	Time constant $10^2 \times k\text{ (s}^{-1}\text{)}$	Half-life (s)	Time constant $10^2 \times k\text{ (s}^{-1}\text{)}$	Half-life (s)			
<i>sacRsacC</i>	1.3 ± 0.23	53 ± 9	1.83 ± 0.08	38 ± 2	1.0	0.69	1.00
<i>sacRasacC</i>	0.33 ± 0.06	210 ± 38	1.21 ± 0.02	57 ± 1	3.4	2.8	4.06
<i>sacRbsacC</i>	0.45 ± 0.06	154 ± 20	2.12 ± 0.15	33 ± 2	2.5	0.8	1.16
<i>sacRcsacC</i>	0.75 ± 0.09	92 ± 11	1.26 ± 0.04	55 ± 2	1.5	0.31	0.45

increase in its stability. The *sacRcsacC* mRNA steady-state level is slightly increased while the production of levanase is decreased. In this case, the SD sequence was introduced at a distance of 171 nt from the RBS in the very first segment of the transcript. It seems that stalling of ribosomes sterically protects the mRNA and therefore delays degradation, as found previously by Bechhofer & Dubnau (1987). The cause of decreased protein production is not obvious. In fact the RNA-binding of SacY, the antiterminator protein, in this region (Crutz *et al.*, 1990) complicated our interpretation of the results obtained.

The last point that deserves discussion concerns the accumulation of an apparently homogeneous short transcript obtained with the various *sacRsacC* fusions. Could this be due to a premature release of the RNA polymerase or to 3′–5′ degradation?

Both events could result from the presence of a secondary structure in this region (Farr *et al.*, 1999). The prediction obtained from the MFOLD program (Zuker *et*

al., 1999) shows that there is a high probability that this region contains two stem-loops, the first being followed by a sequence rich in A and U (Fig. 8). The calculated free energy of these loops is similar to that of the *ρ*-independent terminators of the four proteins. Finally, the decay of the short transcript is faster than that of the full-length transcript while the proportion of the latter is two times lower. We thus favour the hypothesis that the short transcript results from premature release of the mRNA by the polymerase rather than nuclease degradation.

Taken together, the data presented here attribute an important role to the nucleotide sequence of the structural gene in mRNA stability by influencing the cleavage activities of nucleases. These activities are increased or reduced as a function of ribosome binding protection. Thus, an improvement in the production yield is possible by increasing mRNA stability with an additional SD sequence in the 5′ untranslated region. This possibility needs to be addressed in future studies of α -amylase and levansucrase.

Fig. 8. Terminator-like structure of the 3' end of *sacC* short transcript. The secondary structures of the *sacC* short transcript which represents the folding of minimum free energy was obtained by computer prediction using the MFOLD program (Zuker *et al.*, 1999). Nucleotides are laid out in such a way that paired bases are proximal and the open regions surrounded by single-stranded bases are represented as loops. The free energy (ΔG_0) of the stems is indicated.

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