A ribonucleic antiterminator sequence (RAT) and a distant palindrome are both involved in sucrose induction of the *Bacillus subtilis* sacXY regulatory operon

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**INTRODUCTION**

*Bacillus subtilis* can utilize sucrose via both extracellular and intracellular pathways. The first involves a secreted levansucrase encoded by the sucrose-inducible *sacB* gene. Induction of *sacB* is mediated by an antitermination mechanism which appears to be conserved in different systems (Steinmetz, 1993; Bardowski et al., 1994; Le Coq et al., 1995). According to the model proposed by Aymerich & Steinmetz (1992), the stem–loop structure of a ribonucleic antiterminator (RAT) located in the leader region of the *sacB* transcript is stabilized in the presence of sucrose by an antiterminator protein, SacY; the formation of the SacY–RAT complex prevents alternative formation of an overlapping rho-independent transcription terminator, allowing transcription of the downstream *sacB* ORF. In the absence of sucrose, SacY activity is repressed by SacX (Crutz et al., 1990), a negative regulator homologous to the SacP sucrose permease of the phosphotransferase system (Zukowski et al., 1990). This leads to termination of transcription upstream from the *sacB* ORF. The second metabolic pathway requires concomitant permeation and phosphorylation of intracellular sucrose 6-phosphate by a
phosphosucrase encoded by sacA. sacP and sacA constitute an operon whose expression is sucrose-inducible, probably through a similar antitermination mechanism since (i) the sacPA leader region carries a rho-independent transcription terminator overlapping a RAT homologous probably through a similar antitermination mechanism to the RAT, and (ii) sacPA expression is positively regulated by SacT, which is highly homologous to SacY (Arnaud et al., 1992; Le Coq, unpublished results). Although both pathways seem to be regulated by an analogous mechanism, maximal expression of sacB and sacPA is not observed at the same inducer concentration: full sacB expression is induced only by 1% (w/v) sucrose, whereas sacPA is fully induced at 0.1% sucrose (Steinmetz et al., 1989; Steinmetz & Aymerich, 1990).

The sacX and sacY genes are organized in an operon which is subject to at least two regulations. In addition to the DegS-DegU control of transcription initiation, sacXY expression is inducible by sucrose: previous experiments performed with high sucrose concentration showed that this induction was mediated by SacY itself and/or SacT (Cruz & Steinmetz, 1992). Furthermore, the sacXY leader sequence contains a sequence highly homologous to the sacB and sacPA RATs. However, this RAT-like sequence does not appear to overlap any apparent rho-independent transcription terminator. It has been observed that deletion of a large fragment of the leader region, downstream from the RAT-like sequence, leads to a constitutive expression of the operon (Cruz & Steinmetz, 1992). These observations suggested that the induction of sacXY could occur differently from that of sacPA and sacB.

In the work described in this paper, we carried out site-directed mutagenesis to demonstrate the involvement of the RAT-like sequence in sacXY induction. A deletion analysis in the leader region was undertaken to locate the other putative cis-acting regulatory element. We also investigated the contribution of both the SacY and SacT antiterminators to sacXY induction in the presence of the relevant sucrose concentration.

### Table 1. B. subtilis strains used in this study

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>Origin of reference</th>
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<tr>
<td>GM720</td>
<td>sacA321 sacBΔ23 deg U32</td>
<td>Crutz &amp; Steinmetz, 1992</td>
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<tr>
<td>GM755</td>
<td>SP5SA1</td>
<td>This study (Fig. 1)</td>
</tr>
<tr>
<td>GM788</td>
<td>GM720 with sacXYΔ4</td>
<td>This study</td>
</tr>
<tr>
<td>GM820</td>
<td>GM720 with sacY::neo</td>
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<tr>
<td>GM824</td>
<td>GM720 with sacT::neo</td>
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<td>GM836</td>
<td>GM720 with sacXYΔ4 sacT::neo</td>
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</tr>
<tr>
<td>GM1070</td>
<td>GM720 with SP5SA1::pAB110</td>
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<td>GM1083</td>
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### METHODS

**Bacterial strains.** Escherichia coli strains TG1 [supE hsdR5 thy (lac-proAB) F' (traD36 proAB' lacZ' proAB+) mlc205] and C236 [dut-1 ung-1 thi relA1/pCJ105] were used for construction of plasmids and for site-directed mutagenesis. *B. subtilis* strains used in this study are listed in Table 1.

**Genetic techniques and media.** Transformation of *E. coli* and *B. subtilis* was performed by standard methods. *B. subtilis* transformants and transductants were selected on LB solid medium (Miller, 1972) containing chloramphenicol (4 mg l⁻¹) or erythromycin (0.4 mg l⁻¹). *E. coli* transformants were selected on LB plates containing ampicillin (80 mg l⁻¹) or neomycin (10 mg l⁻¹). lacZ transcriptional fusions carried by integrative plasmids were inserted into the SP5SA1 prophage (Aymerich & Steinmetz, 1992) by transformation of the *B. subtilis* GM755 strain (Fig. 1b). Recombinant phages were recovered after thermo-induction and used for transduction as previously described (Podvin & Steinmetz, 1992; Cruz & Steinmetz, 1992), allowing chromosomal integration of the transcriptional fusions at an ectopic locus.

**Plasmids.** pAB110 and derivatives are integrative in *B. subtilis*. pAB110 was derived from pUC8 by replacement of the NarI-PstI fragment (containing the multiple cloning site and part of lacZ) by the NarI–EcoRV fragment of pAMU43 (Cruz & Steinmetz, 1992), carrying the cat gene and a sacX'::lacZ' transcriptional fusion (Fig. 1a). pAB112 and pAB115 are pAB110 derivatives with C6 and C6/G23 mutations in the RAT-like sequence respectively (Fig. 4a). pIC121 was derived from pAB110 by replacement of the EcoRI–BamHI fragment carrying the sacXY leader region, by the EcoRI–BamHI fragment of pTrpB2 (Shimotsu & Henner, 1986) containing the trpE constitutive promoter (Fig. 1c). Insertion of a synthetic XbaI–BamHI fragment, obtained by annealing P4 and P5 complementary oligonucleotides, into pIC121 led to pIC414 (Fig. 1c). A collection of pAB110 derivatives carrying different lengths of the sacXY leader region fused to lacZ' was constructed as follows: insertion of a BamHI neomycin resistance cassette (neo) into pAB110 at the BamHI site led to pAB202; pAB202 was then digested with NdeI (included in the neo sequence) and finally submitted to Bal31 exonuclease. The reaction was stopped at different times and the plasmids were digested with BamHI to remove remaining neo sequences.
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DNA ends were filled by the Klenow fragment of DNA polymerase I and ligated by T4 DNA ligase. The structure of the fusions in the resulting pAB301, pAB302, pAB303 and pAB305 plasmids is shown in Fig. 5. pAB304 was constructed by deleting the SalI–BamHI fragment of pAB110.

**DNA manipulations.** Standard techniques were used for plasmid and phagemid construction. Site-directed mutagenesis was performed by the method of Kunkel (Kunkel et al., 1987) using a Bio-Rad Muta-Gene kit. DNA sequencing of mutations and deletions was performed by the method of Sanger (Sanger et al., 1977), using a Sequenase kit (US Biochemicals). Primers P2 (5' - GCTTGGGATCCATGCATAGGGGACATTTGTTGCA-TTTTTCAC-3') and P5 (5' - CTACAGATCCGATCCTGAGGTCACCGTC-3'), used for pIC414 construction, were synthesized with a Cyclone synthesizer (Biosearch).

**Leader RNA computer analysis.** Optimal secondary structures of the first 182 nucleotides of the sacXY leader RNA were determined using the Zuker's Mfold program from the University of Wisconsin Genetics Computer Group software package (Genetics Computer Group, 1994).

**β-Galactosidase assays.** B. subtilis liquid cultures were grown in LB or CgCH (Crutz et al., 1990) supplemented with tryptophan (50 mg l⁻¹) when required. Preparation of B. subtilis extracts and β-galactosidase assays were carried out as previously described (Miller, 1972; Piggot & Curtis, 1987). Standard deviations were not more than 15% for activities equal to or higher than 3.5 Miller units, and not more than 40% for lower activities. Representative results are given for each assay.

**RESULTS**

**Role of SacY and SacT antiterminators in sacXY induction**

It was shown previously that induction of sacXY in 1% sucrose required either SacY or SacT antiterminators (Crutz & Steinmetz, 1992). SacY allows full sacB expression in 1% sucrose whereas 0-1% is sufficient for full sacPA induction via SacT. We investigated the effect of these two different sucrose concentrations to study the physiological role of SacY and SacT antiterminators in sacXY induction. A sacX′::lacZ′ transcriptional fusion carried by pAB110 (Fig. 2) was inserted at an ectopic locus into GM720 and derivatives carrying deletions of sacY, sacT or both. These recombinant strains were grown in CgCH medium supplemented with sucrose (0-1 or 1%) or not supplemented, and β-galactosidase activity was measured. Experiments with high sucrose concentration (i.e. 1%) confirmed that both antiterminators allowed sacXY induction.

**Fig. 1.** Structure of plasmid pAB110 and derivatives, and utilization of a SPβSA1 prophage as integration platform. (a) pAB110 carries the sacXY leader region (black box) fused to lacZ′ (hatched box). In this construction, spaVG translational signals are cloned between lacZ′ and the sacXY leader sequence. The sacXY RAT-like sequence is overlaid by inverted arrows. (b) SPβSA1 phage contains from left to right, the left end of Tn917 (Tn′), lacZ fused to spaVG translational signals (lacZ2) and the right end of Tn917 (Tn), including the erythromycin resistance gene (erm) (Crutz & Steinmetz, 1992); pAB110 and its derivatives can be inserted into SPβSA1 prophage through in vivo homologous recombination in lacZ sequences. (c) pIC121 was derived from pAB110 by replacing the sacXY leader region by an EcoRI–BamHI fragment of ptrbG1, containing the constitutive trpEp promoter (grey box) (Shimotsu & Henner, 1986). pIC414 was derived from pIC121 by insertion of a synthetic XbaI–BamHI fragment reconstituting the sequence of the sacXY leader region from position 149 to 193 and carrying the palindromic around the Sacl site (Fig. 2).

**Fig. 2.** Sequence of the sacX′::lacZ transcriptional fusion present in pAB110 and extent of the Ba31 deletions. The original sacXY sequence is in capital letters; lower case letters are spaVG sequences (Perkins & Youngman, 1986). sacXY promoter boxes (-35 and -10), transcription start (+1) and spaVG RBS are indicated. The RBS-like sequence and the palindromic are boxed and overlaid by inverted arrows, respectively. Deletions (Δ1, Δ2, Δ3, Δ4, Δ5) extend from the BamHI restriction site towards the bent arrows.
induction, whereas 0.1% sucrose allowed activation of SacT only (Fig. 3). The contribution of each antiterminator did not appear to be equivalent, as the activities observed with 1% sucrose in the sacY and the sacT mutants were about 75% and 30% of that in the wild-type strain, respectively. As expected, the fusion was not inducible at all in the GM1083 sacYsacT double mutant. These results are in accordance with what is known concerning the activation of the antiterminator activities of SacY and SacT for sacB and sacPA induction, respectively (Steinmetz & Aymerich, 1990).

Mutational analysis of the RAT-like sequence upstream from the sacX ORF

Previous experiments on the sacB RAT showed that pairing of some nucleotides in the RAT was involved in sacB induction (Aymerich & Steinmetz, 1992). These results suggested that folding of the sacB RAT in the mRNA leader into a stem–loop structure was essential for induction. A similar conformation can be proposed for the sacXY RAT-like sequence (Fig. 4a). According to this model, the C6 mutation is expected to alter the stem–loop stability (Fig. 4a). In order to test if the proposed secondary structure of the sacXY RAT-like sequence was required for induction, β-galactosidase activities expressed by the wild-type sacX::lacZ fusion (GM1070) and the fusion carrying the C6 mutation in the RAT-like sequence (GM1072) were compared. As shown in Fig. 4(b), the C6 mutation abolished lacZ induction by sucrose. Addition of a G23 compensatory substitution (Fig. 4a), expected to replace the C6/G23 mispairing by the C6/G23 pairing, restored an induction level similar to that of the wild-type (GM1075, Fig. 4b). These results showed that the RAT-like sequence is involved in sacXY induction by sucrose and strongly suggest that pairing of nucleotides at positions 6 and 23 is necessary for sucrose induction.

Disruption of the putative B. subtilis rho gene does not affect sacXY induction

The absence of any rho-independent terminator overlapping the RAT-like sequence of sacXY is a novel feature. We could speculate on the presence of a rho-dependent terminator, which might be more difficult to identify by sequence analysis. A putative B. subtilis rho gene has been cloned (Quirk et al., 1993). To study its involvement in sacXY induction, this gene was inactivated in strain GM1070. No significant differences were found in sacX::lacZ induction levels expressed in GM1070 and the resulting isogenic rho mutant (data not shown). Thus, this putative rho gene does not appear to be involved in sacXY induction.

Involvement of a palindromic sequence

It has been shown previously that a sacX::lacZ fusion with a leader region deleted downstream from the SalI restriction site (Fig. 2) was expressed constitutively (Crutz
The palindromic sequence has a negative effect on expression

To test the effect of the palindrome in an heterologous context, a synthetic fragment was cloned into pIC121, between the trpE promoter and lacZ', to give pIC414 (Fig. 1c). This fragment, obtained by annealing P4 and P5 complementary oligonucleotides, contains the sequence of the sacXY leader region from position 149 to 193 including the palindrome (Fig. 2). Fusions carried by pIC121 and pIC414 were inserted into GM720 and β-galactosidase activities were measured. No significant difference in lacZ expression was observed between the two fusions when the cultures were grown at 37 °C. However, when cells were grown at 20 °C, expression of the pIC414 fusion was significantly decreased as compared to the pIC121 one (85 and 178 Miller units, respectively). These results suggested that the sequence cloned in pIC414 has a negative effect on transcription although it might not include all the cis-acting elements involved in sacXY regulation.

**DISCUSSION**

The *B. subtilis* SacY and SacT antiterminators, involved respectively in sacB and sacPA induction by sucrose, also allow sacXY sucrose induction (Crutz & Steinmetz, 1992). Investigation of these antiterminator activities, under our experimental conditions, showed that SacT mediates sacXY induction at a concentration of sucrose as low as 0.1%, whereas SacY is activated only at a higher concentration. Moreover, SacY-mediated induction at 1% sucrose appears weaker than that mediated by SacT (Fig. 3). However, this SacY-mediated induction was measured in a sacT mutant. In such a context, expression of sacY should be lower than in a wild-type strain, in which constitutive expression of sacT (Arnaud et al., 1992) contributes to sacY expression from low sucrose concentrations. SacY is then present in an inactive form, and can be activated by 1% sucrose. Thus, the effect of SacY in sacXY induction observed in a sacT mutant might not reflect its real contribution in a wild-type strain.

The weak specificity of the sacXY RAT towards SacY and SacT antiterminators is in accordance with known determinants of specificity of the RATs. It was shown previously that changing the nucleotides at position 3 or 13 in the sacB RAT (Fig. 4a) to those present in the sacPA RAT increased sacB induction by SacT without affecting SacY-mediated induction (Aymerich & Steinmetz, 1992). A model was proposed in which the U in position 3 and the A in position 13 of the sacB RAT acted as negative determinants of specificity for interaction with SacT. The nucleotides at these positions in the sacXY RAT are identical to those present in the sacPA RAT. The established interaction of the sacXY RAT with SacY and SacT therefore confirms the model. Cross-talk between the two sucrose catabolic pathways is known to be mediated by SacY and SacT antiterminators (Steinmetz et al., 1989). Here, we demonstrate a clear link between the
sacB and sacPA induction pathways via antiterminator activity of SacT on sacXY expression.

The molecular mechanism of sacXY induction is still unknown. A RAT-like sequence is present in the sacXY leader region. However, contrary to other induction systems of the same family, no apparent overlapping rho-independent terminator has been found. Involvement of this RAT in induction of the operon was established by site-directed mutagenesis. Moreover, these experiments showed that the formation of a secondary structure of the RAT was required. Deletion analysis clearly showed that the induction level of sacXY is not significantly affected unless a palindrome, located 100 nt downstream from the RAT, is at least partially deleted. Thus this palindrome seems to be another cis-acting regulatory element involved together with the RAT-like sequence in sacXY induction.

These data provide evidence for a novel mechanism in which a sequence homologous to the RATs interacts with a distant palindrome. Computer analysis (Genetics Computer Group, 1994) of potential secondary structures of the RNA leader starting at the transcription start and encompassing the palindrome was performed. When no constraint was imposed, the most stable structure of the RNA \( \Delta G = -47 \text{ kcal mol}^{-1} (-196.7 \text{ kJ mol}^{-1}) \) corresponds to the formation of a long imperfect hairpin containing the RAT sequence, and thus preventing the formation of its normal secondary structure; this allows the folding of the palindrome in a stem–loop followed by a stretch of uridine residues (Fig. 6a). In an energy range of 5%, only one other suboptimal secondary structure was found, which was very similar to the optimal one and led also to the folding of the palindrome in a stem–loop (not shown). When secondary-structure-formation of the RAT was forced, the sequences downstream were folded in such a way that the palindrome could not adopt the stem–loop structure (Fig. 6b). The same results were obtained when the complete leader RNA sequence (287 nt) was submitted to the analysis (not shown). This computer analysis could simulate the mechanism by which these two distant elements interact: in the absence of sucrose, the palindrome would spontaneously adopt a stem–loop structure, whereas in the presence of sucrose activated antiterminators could stabilize the RAT structure, preventing the alternative formation of the palindrome stem–loop. This model could explain the weak effect of the palindrome on expression when cloned between the trpEp promoter and the lacZ reporter gene: as suggested by computer analysis, the stem–loop formation of the palindrome could be highly stabilized by secondary-structure-formation of the leader messenger, which cannot occur in the heterologous construction. Thus, in such a context, this secondary structure might be rather unstable at 37 °C, but could still be able to form at 20 °C.

The exact function of the palindrome remains unclear. Looking at its structure and the absence of phenotype conferred by the rho disruption, its involvement as a rho-independent terminator seems likely. However, the existence of another rho gene cannot be excluded; this could explain why rho is not essential in B. subtilis contrary to what is known for E. coli (Das et al., 1976). We cannot either exclude a model in which a differential stability of the mRNA is involved, for example with the palindrome being the target of an endonuclease. Northern analysis could allow clarification of this point, but this would require the detection of sacXY mRNAs transcribed from the wild-type promoter, which was not possible up to now as their level appears to be very low (Tortosa & Le Coq, unpublished results).

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REFERENCES


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