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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The *Bacillus subtilis* *sacXY* regulatory operon is involved in sucrose induction of the levansucrase *sacB* gene by an antitermination mechanism. In the presence of sucrose, the activated SacY antiterminator protein stabilizes the secondary structure of a ribonucleic antiterminator sequence (RAT) located in the leader region of the *sacB* transcript, and overlapping a rho-independent transcription terminator. Formation of the SacY–RAT complex prevents alternative formation of the terminator, allowing transcription of the downstream sequences. In the absence of sucrose, inhibition of SacY activity by SacX leads to termination of transcription. Expression of *sacXY* is also sucrose-inducible. This induction was previously shown to be mediated by SacY itself and/or SacT, another antiterminator involved in induction of genes belonging to a distinct sucrose pathway. These antiterminators are not activated at the same concentration of sucrose. We show here that *sacXY* induction occurs through activation of either SacY or SacT antiterminators, at their respective sucrose activation concentration. This result demonstrates a link between SacY- and SacT-mediated metabolic pathways. In addition, the *sacXY* leader region carries a RAT-like sequence, which however does not appear to overlap any apparent rho-independent transcription terminator. Site-directed mutagenesis experiments on this RAT-like sequence demonstrated its involvement in sucrose induction. Deletions generated in the *sacXY* leader region showed that a palindrome, located 100 nt downstream from the RAT-like sequence, also acts as a *cis*-acting element. Computer analysis of the leader RNA suggested that formation of the secondary structure of the RAT-like sequence and the palindrome could be mutually exclusive.

Keywords: *Bacillus subtilis*, sucrose induction, *sacXY* operon, ribonucleic antiterminator

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 sucrose by SacP, and subsequent hydrolysis of intracellular sucrose 6-phosphate by a

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

Strain	Genotype	Origin of reference
GM720	<i>sacA321 sacBΔ23 deg U32</i>	Crutz & Steinmetz, 1992
GM755	SPβSA1	
GM788	GM720 with <i>sacXYΔ4</i>	
GM820	GM720 with <i>sacY::neo</i>	
GM824	GM720 with <i>sacT::neo</i>	
GM836	GM720 with <i>sacXYΔ4 sacT::neo</i>	
GM1070	GM720 with SPβSA1::pAB110	This study (Fig. 1)
GM1072	GM720 with SPβSA1::pAB112	
GM1073	GM720 with SPβSA1::pAB115	
GM1074	GM788 with SPβSA1::pAB110	
GM1077	GM820 with SPβSA1::pAB110	This study
GM1080	GM824 with SPβSA1::pAB110	
GM1083	GM836 with SPβSA1::pAB110	

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 to the *sacB* RAT, and (ii) *sacPA* expression is positively regulated by SacT, which is highly homologous to SacY (Débarbouillé *et al.*, 1990; 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 % (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 (Crutz & 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 (Crutz & 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.

METHODS

Bacterial strains. *Escherichia coli* strains TG1[*supE hsdΔ5 thi Δ(lac-proAB) F'* (*traD36 proAB⁺ lacI^q lacZΔM15*)] and C[J236 [*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 SPβSA1 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; Crutz & 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 (Vieira & Messing, 1982) by replacement of the *NarI*-*PvuII* fragment (containing the multiple cloning site and part of *lacZ*) by the *NarI*-*EcoRV* fragment of pAMU43 (Crutz & 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 ptrpBG1 (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 *NotI* (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.

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, *spoVG* 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 *spoVG* translational signals (*lacZ'*) 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 *ptrpBGI*, 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 palindrome around the *SalI* site (Fig. 2).

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'-GCTGCCCAGTCAGAATCCCGCCATAT-3') and P3 (5'-ATCATTGGGTCTTCCCTGCCTGCCCA-3'), used for site-

directed mutagenesis, and oligonucleotides P4 (5'-CTAGATTCTCATGCTCCGTCGTCGACGCGGGCATTTTTGTCA-TTTTCAG-3') and P5 (5'-GATCCTG-AAAATGACAAAA-ATGCCCCGTCGTCGACGACGGAGCATGAGAAT-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*

Fig. 2. Sequence of the *sacX'::lacZ* transcriptional fusion present in pAB110 and extent of the *Bal31* deletions. The original *sacXY* sequence is in capital letters; lower case letters are *spoVG* sequences (Perkins & Youngman, 1986). *sacXY* promoter boxes (-35 and -10), transcription start (+1) and *spoVG* RBS are indicated. The RAT-like sequence and the palindrome are boxed and overlaid by inverted arrows, respectively. Deletions (Δ1, Δ2, Δ3, Δ4, Δ5) extend from the *BamHI* restriction site towards the bent arrows.

Fig. 3. β -Galactosidase activities in the *sacX'*::*lacZ*-carrying GM1070 strain and derivatives deleted for *sacY* (GM1074 and GM1077), *sacT* (GM1080) or both (GM1083) genes. Cultures were grown in CgCH supplemented with sucrose (0.1 or 1%) or not supplemented.

induction, whereas 0.1% sucrose allowed activation of SacT only (Fig. 3). The contribution of each anti-terminator 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 *sacY sacT* 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/C23 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.

Fig. 4. Effect of point mutations in the RAT-like sequence of the *sacXY* leader region. (a) Proposed secondary structure for the *sacB* and *sacPA* RATs (Aymerich & Steinmetz, 1992), and for the *sacXY* RAT-like sequence. Rho-independent terminators overlapping the *sacB* and *sacPA* RATs are overlaid by inverted arrows. C6 substitution and C6/G23 double substitution in the *sacXY* RAT-like sequence are indicated. (b) β -Galactosidase activities measured in *B. subtilis* strains carrying a *lacZ* gene fused to the C6 single substituted (GM1072), the C6/G23 double substituted (GM1075) or the wild-type (GM1070) *sacXY* leader region. Cultures were grown in CgCH supplemented with sucrose (0.1 or 1%) or not supplemented.

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 *Sa*I restriction site (Fig. 2) was expressed constitutively (Cruz

Fig. 5. Effect of deletions in the *sacXY* leader region. *lacZ* was fused to different *sacXY* leader fragments. +1 indicates the transcription start site, the RAT-like sequence and the palindrome around the *SalI* site are overlined by inverted arrows and the *lacZ* gene is boxed. Extent of deletions generated by *Bal31* exonuclease ($\Delta 1$, $\Delta 2$, $\Delta 3$ and $\Delta 5$) or by *SalI*-*BamHI* restriction ($\Delta 4$) are indicated by dashed lines; the plasmids carrying each of the deletions are listed. pAB110 and deletion derivatives were introduced by transformation into GM720. Transformants were grown in CgCH medium supplemented or not with sucrose. β -Galactosidase activities were measured for each fusion in the absence (ni, not induced) or in the presence (i, induced) of 1% sucrose and the calculated induction ratio (i/ni) is given.

& Steinmetz, 1992). Deletions upstream from the *sacX* initiation codon, towards the RAT-like sequence, were generated with *Bal31* exonuclease. A set of five transcriptional fusions ($\Delta 1$ - $\Delta 5$), in which different lengths of the leader region remained, was constructed (Fig. 5). These fusions (carried respectively by plasmids pAB301, pAB302, pAB303, pAB304 and pAB305) were inserted into GM720 and β -galactosidase activities were measured in the absence or in the presence of 1% sucrose. As shown in Fig. 5, fusions carried by pAB110, pAB301, pAB302 and pAB303 led to similar induction levels, although there were some variations in the β -galactosidase activities. These differences in general expression might be due to the artificial contexts created by these constructions, which could affect transcription efficiency and/or mRNA stability. The two other fusions (carried by pAB304 and pAB305) in which a palindromic sequence had been partly or completely deleted were, respectively, slightly and not inducible at all by sucrose. This suggested that the palindrome is involved, together with the RAT-like sequence, in the mechanism of induction of the *sacXY* operon by sucrose.

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*p constitutive 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 *trpE* promoter and the *lacZ* reporter gene: as suggested by computer analysis, the stem-loop form 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

Fig. 6. Optimal secondary structures proposed by the Zuker's MFold program (Genetics Computer Group, 1994) for the first 182 ribonucleotides of the *sacXY* leader RNA. The RAT-like sequence and the palindrome are boxed and overlined by inverted arrows, respectively. (a) Most stable RNA secondary structure [$\Delta G = -47 \text{ kcal mol}^{-1}$ ($-196.7 \text{ kJ mol}^{-1}$)] without constraint. (b) Most stable RNA secondary structure [$\Delta G = -41.1 \text{ kcal mol}^{-1}$ ($-172.0 \text{ kJ mol}^{-1}$)] forcing the formation of the RAT stem-loop.

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

- Arnaud, M., Vary, P., Zagorec, M., Klier, A., Débarbouillé, M., Postma, P. & Rapoport, G. (1992). Regulation of the *sacP.A* operon of *Bacillus subtilis*: identification of phosphotransferase system components involved in SacT activity. *J. Bacteriol* **174**, 3161–3170.
- Aymerich, S. & Steinmetz, M. (1992). Specificity determinants and structural features in the RNA target of the bacterial anti-terminator proteins of the BglG/SacY family. *Proc. Natl. Acad. Sci. USA* **89**, 10410–10414.
- Bardowski, J., Ehrlich, S. D. & Chopin, A. (1994). BglR protein, which belongs to the BglG family of transcriptional anti-terminators, is involved in β -glucoside utilization in *Lactococcus lactis*. *J. Bacteriol* **176**, 5681–5685.
- Crutz, A.-M. & Steinmetz, M. (1992). Transcription of the *B. subtilis* *sacXY* operon, encoding regulators of sucrose metabolism, is both sucrose inducible and controlled by the DegS/DegU signalling system. *J. Bacteriol* **174**, 6087–6095.
- Crutz, A.-M., Steinmetz, M., Aymerich, S., Richter, R. & Le Coq, D. (1990). Induction of levansucrase in *Bacillus subtilis*: an anti-termination mechanism negatively controlled by the phosphotransferase system. *J. Bacteriol* **172**, 1043–1050.
- Das, A., Court, D. & Adhya, S. (1976). Isolation and characterization of conditional lethal mutants of *Escherichia coli* defective in transcription termination factor rho. *Proc. Natl. Acad. Sci. USA* **73**, 1959–1963.
- Débarbouillé, M., Arnaud, M., Fouet, A., Klier, A. & Rapoport, G. (1990). The *sacT* gene regulating the *sacP.A* operon in *Bacillus subtilis* shares strong homologies with transcriptional anti-terminators. *J. Bacteriol* **172**, 3966–3973.
- Genetics Computer Group (1994). *Program Manual for the GCG Package*, Version 8, August 1994. Madison, Wisconsin: Genetics Computer Group.
- Kunkel, T. A., Roberts, J. D. & Zakour, A. R. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* **154**, 367–382.
- Le Coq, D., Lindner, C., Krüger, S., Steinmetz, M. & Stülke, J. (1995). New β -glucoside (*bgl*) genes in *Bacillus subtilis*: the *bglP* gene product has both transport and regulatory functions similar to those of BglF, its *Escherichia coli* homolog. *J. Bacteriol* **177**, 1527–1535.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Perkins, J. B. & Youngman P. J. (1986). Construction and properties of Tn917-*lac*, a transposon derivative that mediates transcriptional gene fusions in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **83**, 140–144.
- Piggot, P. J. & Curtis, C. A. M. (1987). Analysis of the regulation of gene expression during *Bacillus subtilis* sporulation by manipulation of the copy number of *spo-lacZ* fusions. *J. Bacteriol* **169**, 1260–1266.
- Podvin, L. & Steinmetz, M. (1992). A *degU*-containing SP β prophage complements superactivator mutations affecting the *degS* operon. *Res. Microbiol* **143**, 559–567.
- Quirk P. G., Dunkley E. A., Lee P. & Krulwich T. A. (1993). Identification of a putative *Bacillus subtilis* *rho* gene. *J. Bacteriol* **175**, 647–654.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-termination inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
- Shimotsu, H. & Henner, D. J. (1986). Construction of a single copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. *Gene* **43**, 84–94.
- Steinmetz, M. (1993). Carbohydrate catabolism: enzymes, pathways and evolution. In *Bacillus subtilis and other Gram-Positive Bacteria: Biochemistry, Physiology and Molecular Genetics*, pp. 157–170. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Steinmetz, M. & Aymerich, S. (1990). *Bacillus subtilis* *sac deg* constellation: how and why? In *Genetics and Biotechnology of Bacilli*, vol. 3, pp. 303–311. Edited by M. M. Zukowski, A. T. Ganesan & J. A. Hoch. New York: Academic Press.
- Steinmetz, M., Le Coq, D. & Aymerich, S. (1989). Induction by sucrose of saccharolytic enzymes in *Bacillus subtilis*: evidence for two partially interchangeable pathways. *J. Bacteriol* **171**, 1519–1523.
- Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.
- Zukowski, M. M., Miller, L., Cogswell, P., Chen, K., Aymerich, S. & Steinmetz, M. (1990). Nucleotide sequence of the *sacS* locus of *Bacillus subtilis* reveals the presence of two regulatory genes. *Gene* **90**, 153–155.

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