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Transcription of the *Bacillus subtilis sacX* and *sacY* Genes, Encoding Regulators of Sucrose Metabolism, Is Both Inducible by Sucrose and Controlled by the DegS-DegU Signalling System

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The adjacent *sacX* and *sacY* genes are involved in sucrose induction of the *Bacillus subtilis sacB* gene by an antitermination mechanism. *sacB*, encoding the exoenzyme levansucrase, is also subject to regulation by the DegS-DegU signalling system. Using *sacXY'-lacZ* and *sacX'-lacZ* fusions, we show that the transcription of the *sacX* and *sacY* genes is both inducible by sucrose and regulated by DegU. *sacX* and *sacY* appear to constitute an operon, since the deletion of the *sacX* leader region abolished the expression of a *sacXY'-lacZ* fusion. The *degU*-dependent promoter was located by deletion analysis and reverse transcriptase mapping 300 nucleotides upstream from the *sacX* initiator codon. Sucrose induction of the *sacX'-lacZ* fusion requires either SacY or the homologous SacT antiterminator, which is involved in sucrose induction of the intracellular sucrase gene (*sacPA* operon). Sequence analysis of the *sacX* leader region revealed (20 nucleotides downstream from the transcription start site) a putative binding site for these regulators; however, no structure resembling a rho-independent terminator could be found overlapping this site, unlike the situation for *sacPA* and *sacB*. Deletion of a segment of the leader region located 100 nucleotides downstream from this site led to constitutive expression of the *sacXY'-lacZ* and *sacX'-lacZ* fusions. These results suggest that the mechanism of sucrose induction of *sacXY* is different from that of *sacPA* and *sacB*.

Levansucrase, encoded by *sacB*, is a *Bacillus subtilis* extracellular enzyme involved in the metabolism of sucrose. Its synthesis is induced by sucrose through an antitermination mechanism requiring the *sacY* (formerly *sacS*) gene product, which prevents early termination at a rho-independent terminator located upstream of *sacB* (2, 25, 28). Genetic evidence has suggested that SacY activity is modulated by sucrose through a regulatory cascade involving the phosphotransferase system and the *sacX* gene product (5). The *sacX* and *sacY* genes are contiguous and probably constitute an operon; sequence analysis has not revealed any obvious terminator in the intergenic region (38), which moreover appears to be permeable to transcription (13). The deduced amino acid sequence of SacY (38) shows extensive similarity to those of the *B. subtilis sacT* and *Escherichia coli bglG* gene products, involved, respectively, in induction of the *sacPA* operon (encoding a sucrose transporter and an intracellular sucrase) and induction of the *bgl* operon (6, 16, 23); SacT and BglG, activated by the presence of the corresponding inducer, prevent termination at a terminator(s) located upstream of the structural genes (14, 16). Direct evidence was obtained in vitro that BglG is an RNA binding protein; its target sequence is approximately 30 nucleotides (nt) long and overlaps the transcription terminator (10). Binding of BglG to this sequence, referred as RAT (ribonucleic antiterminator), and formation of the terminator hairpin appear to be mutually exclusive. A similar RAT sequence and its position relative to the respective terminator are conserved upstream of *sacPA* and *sacB* (3, 6, 30). Mutational analysis has indicated that SacT and SacY indeed interact with the RAT sequences of *sacPA* and *sacB*, respectively (3). Hence, the mechanism of induction appears to be very similar in these three carbohydrate utilization systems.

In addition to sucrose induction, expression of *sacB* in *B. subtilis* is affected by the *degS-degU* two-component regulatory system (formerly *sacU*); this signalling system has a pleiotropic regulatory role (8, 12). DegU, the effector of the pair, is a positive regulator that, directly or indirectly, activates transcription of *sacB*; *degU*(Hy) and *degU*(−) mutations considerably increase and decrease *sacB* transcription, respectively (1, 25). A target sequence for this DegU-mediated regulation has been localized upstream from the *sacB* promoter (7). However, another target sequence, located downstream from the promoter, has also been identified and appears to be merged with sequences involved in sucrose control (7). This finding suggested that DegU could additionally control *sacB* via the control of *sacY* or its product.

In this report, we show that the transcription of *sacX* and *sacY* is subject to double control by the DegS-DegU system and by sucrose and present a preliminary characterization of the mechanism of induction by sucrose.

MATERIALS AND METHODS

Strains and prophages. *E. coli* TGI [*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)] was used as the host for the construction of plasmids. Isogenic derivatives of *B. subtilis* GM671 and GM720 were constructed as summarized in Table 1. Insertion of the *sacXY*Δ*4* allele (a deletion of the 3′ end of *sacX* and all of *sacY*) into the chromosome by transformation with pSL132 was done as previously described (5). The same method was used to insert the following alleles: *degU*::*neo*, an insertion of a neomycin resistance cassette into *degU*; *sacT*::*neo*, a deletion of all of

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TABLE 1. *B. subtilis* strains

Strain	Genotype[a]	Origin or reference[b]
PY480	SPβ*c2*Δ*2*::Tn*917*	20
GM108	*sacA321* Δ(*sacB*)::*nptI trpC2*	Laboratory collection
GM671	*sacA321 sacB*Δ*23*	Laboratory collection
GM699	SPβMS1	This study (Fig. 1b)
GM719	*sacA321 sacB*Δ*23 degU*::*neo*	pIC34 tf GM671
GM720	*sacA321 sacB*Δ*23 degU32*	Laboratory collection
GM755	SPβSA1	This study (Fig. 1c)
GM788	GM720 with *sacXY*Δ*4*	pSL132 (5) tf GM720
GM820	GM720 with *sacY*::*neo*	pSL190 tf GM720
GM824	GM720 with *sacT*::*neo*	pIC97 tf GM720
GM836	GM720 with *sacXY*Δ*4 sacT*::*neo*	pSL132 (5) tf GM824
GMT693	*sacA321 sacB*Δ*23 degU32 zii-83*::Tn*917*	Laboratory collection
CAM90	GM671(pREP43)	pREP43 tf GM671
CAM91	CAM90 with *degU*::*neo*	GM719 DNA tf CAM90
CAM93	CAM90 with *degU32 zii-83*::Tn*917*	GMT693 DNA tf CAM90

[a] Δ(*sacB*)::*nptI* (formerly *sacR*::*nptI*) is the replacement of *sacB* by the *nptI* kanamycin resistance gene (2); *sacB*Δ*23* [formerly Δ(*sacB-sacR*)*23*] removes the leader region and the 5′ end of the coding sequence of *sacB* (30).

[b] tf, transformation of (the indicated donor plasmid or chromosomal DNA was used to transform the indicated recipient strain).

sacT, except the first 45 and the last 4 codons; and *sacY*::*neo*, a deletion of all of *sacY*, except the first 58 codons. CAM93, a *degU32* mutant harboring pREP43, a replicative plasmid, was constructed by cotransfer of the *degU32* mutation with the *zii-83*::Tn*917* insertion (32), conferring erythromycin resistance; cotransfer of *degU32* was verified on LB plates supplemented with skim milk (8).

SPβrX0 (19), SPβMS1 (27), and SPβSA1 (3) are derivatives of the SPβ*c2*Δ*2*::Tn*917* prophage (20), differing from SPβ*c2*Δ*2*::Tn*917* by sequence substitutions within the inserted Tn*917* (Fig. 1). *lacZ* fusions used in this study were inserted into SPβMS1 or SPβSA1 as shown in Fig. 1 and transduced into various recipient strains as described below. The SPβMS1 prophage (Fig. 1b) can be used as an insertion platform for *lacZ* fusions (initially designed to be inserted into the *amyE* gene) carried by ptrpBG1 derivatives (24).

Plasmids. Several plasmids carrying transcriptional fusions between various fragments of the *sacX-sacY* locus and *lacZ* (fused to the *spoVG* translational signals) were constructed; they were derived from pSL150 (5) as shown in Fig. 2; all are integrative, except for pREP43. pAMS20 is pAMU36 in which the *Eco*RI-*Bam*HI fragment upstream of *lacZ* has been replaced by the *Eco*RI-*Sal*I fragment of pAMU43, containing part of the *sacX* upstream region (Fig. 2).

pIC34 contains a neomycin resistance gene (*neo*) inserted in the *degU* gene; it was derived from pDH74 (8) by insertion of a *Sma*I fragment from pBEST501, containing *neo* (11), at the *Hpa*I site in *degU*. pIC97 and pSL190 are integrative plasmids carrying, respectively, the *sacT* and *sacY* genes, a fragment in each of which (*Bgl*II for *sacT* and *Asu*II-*Nru*I for

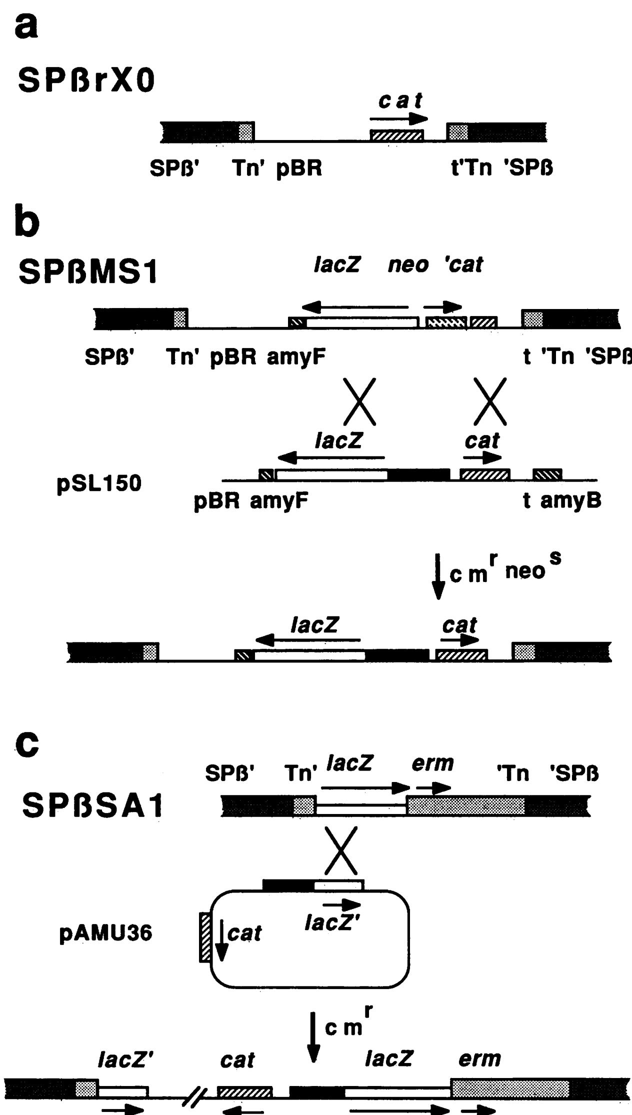

FIG. 1. Structures of the SPβrX0, SPβMS1, and SPβSA1 prophages and utilization of SPβMS1 and SPβSA1 as insertion platforms for *lacZ* fusions. These derivatives of SPβ*c2*Δ*2*::Tn*917* were constructed in several in vivo recombination steps affecting the inserted Tn*917* transposon. (a) SPβrX0 was previously described (19) and contains, from left to right, the left end of Tn*917* (Tn′), including the 5′ end of the *erm* gene; pBR322 sequences (pBR); the *cat* gene, a 276-bp fragment from pBR322 (t); and the right end of Tn*917* (′Tn). The Tn′-pBR-*cat*-t-′Tn segment is from a pTV21Δ2 (34) derivative carrying a 1.9-kb *Bgl*II deletion within ′Tn. (b) SPβMS1 was derived from SPβrX0 in two transformation steps (27), which substituted *amyF-lacZ-neo* sequences for the 5′ end of the *cat* gene in SPβrX0 (*amyF*, the "*amy*-front" segment of the *amyE* gene, and the promoterless *lacZ* gene are from plasmid ptrpBG1 [24]; the *neo* cassette is from pBEST501 [11]; and *′cat* is the 3′ end of the *cat* gene with a deletion to the *Nco*I site). Derivatives of ptrpBG1 or pSL150 (shown linearized at the *Sca*I site) can be inserted into SPβMS1 through a double crossover, as shown. These insertions can be distinguished from insertions into other loci (for example, *amyE*) by their chloramphenicol-resistant, neomycin-sensitive (Cm^r Neo^s), and amylase-positive phenotype. The black rectangle represents the DNA segment controlling the expression of *lacZ*. (c) SPβSA1 (3) was derived from SPβ*c2*Δ*2*::Tn*917* through substitution of the Tn*917-lac* transposon present in pTV32 (34) for the wild-type Tn*917* transposon. Plasmids such as pAMU36 can be inserted into the prophage by a crossover between the prophage *lacZ* gene and the truncated *lacZ′* gene present in pAMU36. Symbols are as defined for panels a and b.

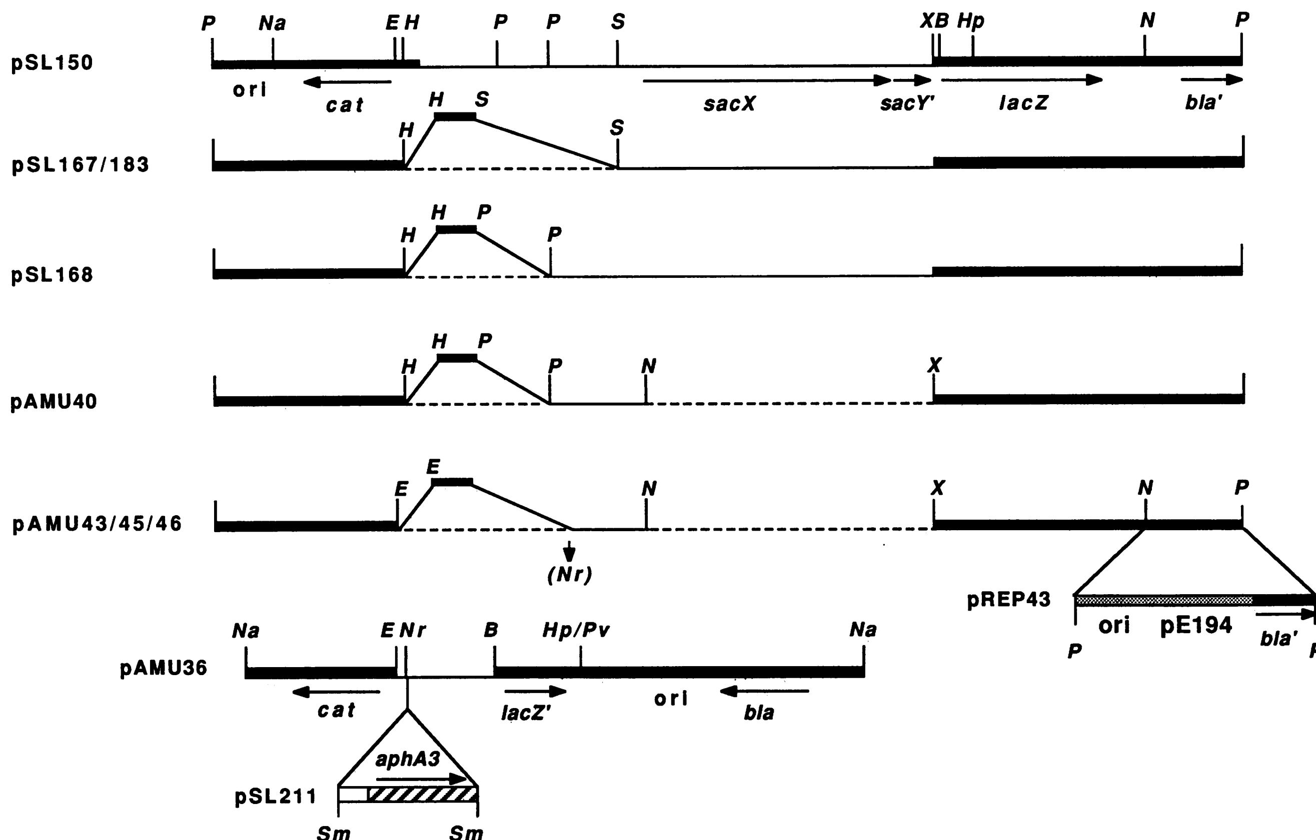

FIG. 2. Construction of *lacZ* fusions. pSL150 and its derivatives are shown linearized at the *Pst*I site within the *bla* gene. pSL150 contains a 3-kb fragment (thin line) from the *B. subtilis* chromosome upstream from a promoterless *lacZ* gene. Regions deleted in pSL150 derivatives are indicated by broken lines. The deletions in pSL167 and pSL168 were obtained by substituting the indicated segment of the pUC19 polylinker (33) for the *Hin*dIII-*Sal*I and *Hin*dIII-*Pst*I fragments of pSL150, respectively. pSL183 is identical to pSL167, except that it carries the *sacXp3* mutation. Construction of pAMU40 was made possible by the replacement of the TTG *sacX* initiation codon by an ATG codon, which created an *Nsi*I restriction site (14). pAMU40 was derived from the pSL168 derivative containing this mutation by intramolecular deletion. pAMU43, pAMU45, and pAMU46 are pAMU40 derivatives containing deletions of the *sacX* leader region (see Fig. 5). The 7-bp linker between *cat* and *B. subtilis* sequences is from pBEST502 (11); the junction created an *Nru*I site in the case of pAMU46. pREP43 was derived from pAMU43 by the introduction of the *cop-6* origin of replication of pE194 carried on a *Pst*I fragment from pHVΔ (35). pAMU36 is shown linearized at its unique *Nar*I site; the *Nar*I-*Hpa*I moiety containing *cat* and the fusion was from pAMU46, and the *Pvu*II-*Nar*I moiety was from pUC19 (33). pSL211 was derived from pAMU36 by insertion of the *Sma*I fragment of pIC28 (36), containing the *aphA3* gene with its promoter but devoid of its transcription terminator, into the *Nru*I site. Only the relevant sites are indicated; *B*, *E*, *H*, *Hp*, *N*, *Na*, *Nr*, *P*, *Pv*, *S*, *Sm*, and *X* represent *Bam*HI, *Eco*RI, *Hin*dIII, *Hpa*I, *Nsi*I, *Nar*I, *Nru*I, *Pst*I, *Pvu*II, *Sal*I, *Sma*I, and *Xba*I restriction sites, respectively.

sacY) has been replaced by the pBEST501 *neo* gene in the same orientation (14).

Genetic techniques and media. Techniques for the use of SPβ were essentially those previously described (21, 37). SPβ lysate was prepared from the relevant lysogenic strains grown in LB medium to the mid-exponential phase and stored at −20°C in 60% glycerol. *B. subtilis* was transduced by mixing 100 μl of an exponentially growing culture in LB medium with 0.2 to 10 μl of SPβ lysate; the mixture was incubated for 5 min at 37°C and then plated. *E. coli* and *B. subtilis* were transformed as previously described (1, 2, 5). Transformants and transductants were selected on LB plates containing the appropriate antibiotics: ampicillin, 50 μg/ml; chloramphenicol, 4 μg/ml (*B. subtilis*) or 15 μg/ml (*E. coli*); erythomycin, 0.4 μg/ml; kanamycin, 5 μg/ml (*B. subtilis*) or 15 μg/ml (*E. coli*); and neomycin, 10 μg/ml.

Liquid cultures of *B. subtilis* were made with CgCH medium, which is C mineral medium (1) supplemented with glucitol (1 g/liter) and casein hydrolysate (0.1 g/liter). This medium was supplemented with tryptophan (50 mg/liter) when required.

DNA manipulation. Plasmid DNA from *E. coli* and chromosomal DNA from *B. subtilis* were extracted as previously described (2, 5). DNA sequences were determined by the method of Sanger et al. (22) with a Sequenase kit (U.S. Biochemical Corp.).

β-Galactosidase assays. Liquid cultures and preparation of extracts were done as previously described (1); induction was performed by the addition of sucrose (20 g/liter). β-Galactosidase activity was assayed as described by Miller (17).

Primer extension analysis. RNA was extracted from cells grown in 10 ml of CgCH medium containing chloramphenicol (10 μg/ml); at an optical density at 600 nm of approximately 1.2, cells were harvested by centrifugation at 4°C and resuspended in 1 ml of CgCH medium. To the suspension were added 1.5 ml of phenol, 60 μl of 20% sodium dodecyl sulfate, and 2.3 g of glass beads (diameter, 0.10 to 0.11 mm; Braun Sciencetec), and the cells were broken by vortexing.

After centrifugation, the aqueous phase was collected and extracted successively with 1 volume each of phenol-chloroform and chloroform. NaCl was added to 0.2 M, and nucleic acids were precipitated with 2.5 volumes of ethanol for 30 min at −20°C. The pellet was recovered by centrifugation and resuspended in 20 mM EDTA. A final saline precipitation with 2 volumes of sodium acetate (4.5 M, pH 7) removed most of the contaminant DNA.

The promoter was mapped with reverse transcriptase by a method adapted from that of Morrisson and Jaurin (18). Oligonucleotides were synthesized with a Cyclone synthesizer (Biosearch). RNA (45 μg) and primer (10 ng) were mixed in a final volume of 10 μl of annealing buffer (0.3 M NaCl, 10 mM Tris-HCl [pH 7.5], 2 mM EDTA). The sample was heated for 5 min at 75°C, cooled slowly to 42°C, and incubated for another 15 min at 42°C. The sample was brought to a final volume of 50 μl in 60 mM NaCl–50 mM Tris-HCl (pH 8.3)–75 mM KCl–3 mM $MgCl_2$–10 mM dithiothreitol–30 U of RNasin–0.5 mM each dCTP, dGTP, and dTTP–10 μCi of [α-^{35}S]dATP; extension of the annealed primer was performed with 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). After incubation for 20 min at 37°C, cold dATP was added to 2 mM, and incubation was continued for another 20 min. The reaction was stopped with 2 μl of 0.25 M EDTA, and RNA was hydrolyzed by the addition of 3.5 μl of 2 N NaOH and incubation at 65°C. After neutralization, DNA was recovered by precipitation, dried, and resuspended in 6 μl of formamide dye solution. Extension products (2 μl) were analyzed on 6% polyacrylamide sequencing gels.

Nucleotide sequence accession number. The nucleotide sequence reported here will appear in the GenBank nucleotide data bank under accession number M97301.

RESULTS

Organization and expression of the *sacX* and *sacY* genes. The regulation of transcription of the *sacX* and *sacY* genes was studied with transcriptional fusions to *lacZ*. To determine whether these genes were organized in an operon, we constructed fusions in which a promoterless *lacZ* gene was fused to various fragments of the *sacX-sacY* locus. The fusions were inserted into the SPβMS1 prophage and transduced into strain GM671 and isogenic strains harboring a *degU* null allele (*degU*::*neo*) or a *degU* hyperactive allele (*degU32*). β-Galactosidase activity was assayed after cultivation of the resulting strains in the presence or in the absence of sucrose. Transcription of *sacY*, as assessed with fusion I, was very low in the *degU*::*neo* mutant and about 20-fold higher in the *degU32* mutant (Fig. 3). Transcription in the wild-type strain was barely higher than that in the *degU*::*neo* mutant (data not shown). In the *degU32* mutant, transcription of *sacY* was increased by approximately 10-fold by sucrose. Deletion of a *Pst*I-*Sal*I fragment upstream from *sacX* (fusion II) abolished the expression of *lacZ*; on the other hand, the expression of *lacZ* from fusion III, in which the coding sequences of *sacX* and *sacY* were deleted, was similar to that from fusion I. This result suggested that *sacX* and *sacY* were transcribed from the same promoter region and that no additional promoter or target for regulation was present downstream from the *sacX* initiator codon.

Thus, the transcription of *sacX* and *sacY* is both inducible by sucrose and controlled by DegU. The genes appeared to be transcribed from a promoter that initiated transcription at a very low rate unless activated by DegU. Sequences upstream from the *Sal*I site (itself 122 nt upstream from the putative initiator codon of *sacX*) were required for detectable transcription of *sacY* (and, as shown below, *sacX*).

Functional analysis of a *degU*-dependent promoter region upstream from *sacX*. The regulatory region upstream from *sacX* was sequenced (Fig. 4). To localize *cis*-acting sequences mediating control by DegU, we constructed a set of *lacZ* fusions, derived from fusion III (Fig. 3), in which progressive deletions were generated from the *Pst*I site toward the *Sal*I site (Fig. 4). The fusions were inserted into SPβMS1 and transduced into strains GM719 (*degU*::*neo*) and GM720 (*degU32*), in which their expression was compared (Table 2). Deletion ΔA, which contains 399 bp of the regulatory region upstream from *lacZ*, did not affect the transcription of *sacX*, whereas deletion ΔB, which removes an additional 57-bp segment, greatly reduced the β-galactosidase activity measured in the *degU32* mutant; sequences mediating *degU* regulation are presumably present within this 57-bp segment. A further 34-bp deletion (ΔC) abolished transcription in the *degU32* mutant. Examination of the DNA sequence upstream from *sacX* revealed a putative *B. subtilis* σ^A promoter (Fig. 4). Deletion ΔC extends past the −35 box.

To determine whether transcription initiated within this region, we used primer extension analysis. To increase the relevant mRNA copy number per cell, we constructed *degU32* and *degU*::*neo* mutants harboring the *sacX* regulatory region on replicative plasmid pREP43, yielding CAM93 and CAM91, respectively. pREP43 carries a high-copy-number origin of replication (*cop-6*) from pE194 (9) and contains the *lacZ* reporter gene fused to the *sacX* regulatory region. As regulation is often altered when *cis*-regulatory targets are present in multiple copies, *lacZ* regulation in these strains was analyzed. We observed that stimulation by *degU32* was not altered and that the high copy number of the fusion indeed led to an increase in *lacZ* expression; however, induction was partly lost (Table 2). RNA was extracted from uninduced CAM93 and CAM91, and extension was performed with primer AM2. A transcription start site was identified at an appropriate distance from the putative promoter. In both strains, the corresponding extension product migrated at the same position, but the signal was more intense with RNA extracted from CAM93 (Fig. 5). Omission of the primer from the reaction resulted in no product being formed. A product migrating at the same position was also obtained when extension was performed with CAM93 RNA and another primer (AM5; Fig. 4) (data not shown). In experiments with AM2, an additional major extension product, 44 nt shorter, was reproducibly observed. This product might correspond to an artifact due to mispolymerization resulting from secondary structures of the transcript (Fig. 5).

Targets for *degU* regulation and sucrose induction of *sacXY*. Fusion IV was derived from fusion ΔA by deletion of a 120-bp segment just upstream from *sacX*. Expression of this fusion was stimulated by *degU32*. However, fusion IV in the *degU32* background was similarly expressed in the absence or the presence of sucrose and at a level corresponding to that of induced fusion ΔA (Fig. 3). This constitutive expression shows that a *cis*-acting region having a negative effect on sucrose induction is located in the 120-bp fragment directly upstream from the *sacX* coding sequence.

The *sacX* promoter was replaced by the strong constitutive *aphA3* promoter to determine whether, as seemed likely, induction by sucrose did not affect transcription initiation. The expression of fusion V, a tripartite fusion among the *aphA3* promoter, the *sacX* leader region, and *lacZ*, was indeed inducible by sucrose (Fig. 3). However, the

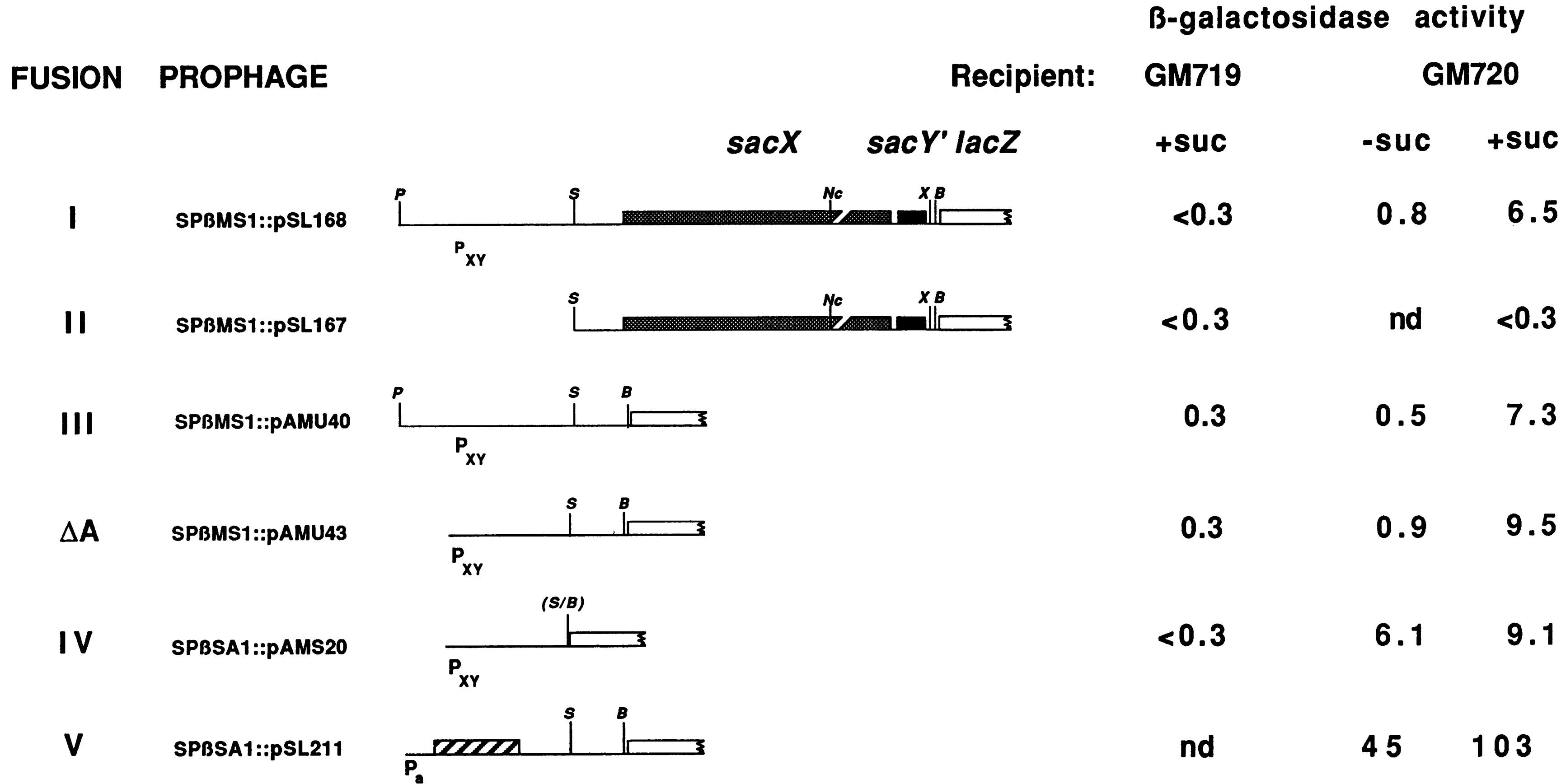

FIG. 3. Expression of *lacZ* fused downstream from various fragments of the *sacX-sacY* locus. Fusions carried by prophages were transduced into GM719 (*degU::neo*) or GM720 (*degU32*). The basal (−suc) and sucrose-induced (+suc) levels of β-galactosidase activity are expressed in Miller units; nd, not determined. Cross-hatched, black, white, and hatched boxes represent *sacX*, *sacY*, *lacZ*, and *aphA3* coding regions, respectively. P_{XY} and P_a indicate the *sacX* promoter characterized in this study and the *aphA3* promoter, respectively. *B*, *Nc*, *P*, *S*, and *X* indicate *Bam*HI, *Nco*I, *Pst*I, *Sal*I, and *Xba*I restriction sites, respectively.

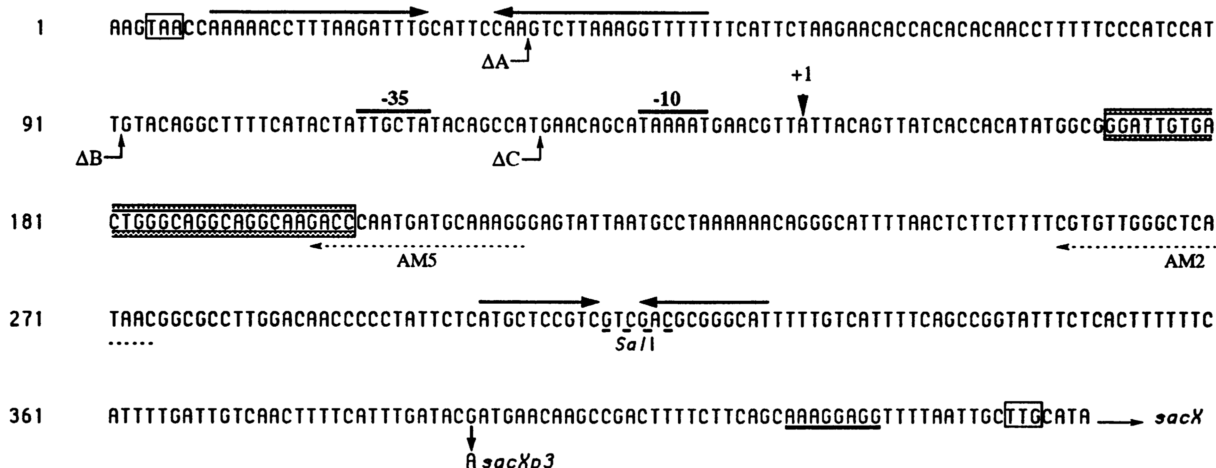

FIG. 4. *sacX* regulatory region. The sequence presented is the junction between the previously published sequences of *epr* (4, 26) and *sacXY* (38). The *sacXp3* mutation is indicated. Inverted repeats of the *epr* terminator and a putative terminator around the *Sal*I site are overlined by arrows. The translational stop codon of *epr* and the start codon of *sacX* are boxed. The putative ribosome binding site of *sacX* is underlined. Deletion endpoints generated by *Bal*31 exonuclease digestion in plasmids pAMU43, pAMU45, and pAMU46 are indicated (ΔA, ΔB, and ΔC, respectively). The vertical arrow indicates the transcription start site (+1). The corresponding −10 and −35 regions are overlined. The positions of oligonucleotides AM2 and AM5, used in primer extension analysis, are indicated. The dotted box highlights a putative binding site for SacY and SacT antiterminators (see Fig. 6). The sequence downstream from *epr*, up to the *Sal*I site, has been deposited with GenBank under accession number M22407 by Sloma et al. (26); it is identical to that presented here, except that it contains an additional undetermined nucleotide between positions −3 and −2.

level of induction was lower than that observed with fusion ΔA in SPβMS1. A similar phenotype was observed with CAM93, which carries fusion ΔA on the pREP43 multicopy plasmid (Table 2). This result could have been due to the titration of a regulator involved in sucrose induction. These experiments showed that *degU* regulation and sucrose inducibility act at different (at least partially separable) targets upstream from *sacX*.

Regulators involved in induction by sucrose of *sacX*. SacY and SacT, regulators of sucrose metabolism, could be involved in induction by sucrose of *sacX*. To test this possibility, we analyzed the induction of fusion III in mutants affected in *sacY*, *sacT*, or both genes as follows. Fusion III was transduced into isogenic derivatives of GM720; deletion of either *sacY* or *sacT* did not have a strong effect, whereas deletion of both abolished induction of the fusion (Table 3). Thus, both antiterminators can induce the fusion in the presence of sucrose, but either of them alone appears to be sufficient.

TABLE 2. Deletion analysis of a *degU*-dependent promoter region upstream from *sacX*

Fusion	Prophage or plasmid	Junction[a]	β-Galactosidase activity[b] in the following genetic background[c]:	
			degU::*neo*	*degU32*
III	SPβMS1::pAMU40	−220	0.3	7.3
ΔA	SPβMS1::pAMU43	−112	0.3	8.4 (0.4)
ΔB	SPβMS1::pAMU45	−55	0.4	1.5
ΔC	SPβMS1::pAMU46	−21	<0.3	<0.3
ΔA	pREP43	−112	7.0	150.0 (70.0)

[a] 5′ End of the *sacX* leader region fused to *lacZ*, given in nucleotides relative to the transcription start site determined in this study (Fig. 4).

[b] In Miller units; measured in both sets of *degU* mutant strains after induction by sucrose, except for values in parentheses (uninduced).

[c] SPβMS1 derivatives were transduced into GM719 and GM720. Mutants carrying pREP43 (last line) were constructed by introduction of *degU*::*neo* or *degU32* into CAM90 (yielding CAM91 and CAM93, respectively).

Characterization of the *sacXp3* mutation. Several classes of mutations affecting in the *sacX*-*sacY* locus have been described and mapped (5, 15). Point mutations rendering SacY sucrose independent have been sequenced (5). Another class of mutations leads to overproduction of levansucrase in the presence of sucrose; these mutations were mapped within the upstream portion of the operon and might affect its regulation (5). We cloned the *sacXp3* allele (formerly *sacS*^{h}*3*) and determined the nucleotide sequence of the segment in which the mutation lay. This mutation corresponds to a point mutation at position +244 (Fig. 4); the G-to-A transition transformed a TACGAT sequence into a TACAAT sequence, closer to the consensus of a σ^A promoter −10 box. A putative −35 box (TTGATT) lay 17 bp upstream. To test whether the *sacXp3* mutation indeed created a promoter, we constructed pSL183, which is identical to pSL167 (Fig. 2) except that it carries this mutation. The fusions carried by pSL167 and pSL183 were inserted into the chromosome of a *degU*$^+$ strain (GM108) by the method of Shimotsu and Henner (24). Expression of the pSL183 fusion was high and similar in the absence and in the presence of sucrose (10.5 and 8.6 Miller units, respectively), whereas expression of the pSL167 fusion was undetectable, as shown for fusion II in Fig. 3.

DISCUSSION

The *sacX*-*sacY* locus was previously shown to consist of two genes, both involved in induction of *sacB* by sucrose (5, 38). It is flanked by transcription terminators separating it from the upstream *epr* gene (4, 26) and a downstream open reading frame in the opposite orientation (38). *sacX* and *sacY* both appeared to be transcribed from a region upstream from *sacX* (fusions I, II, and III; Fig. 3); these results and the observations discussed below indicated that *sacX* and *sacY*

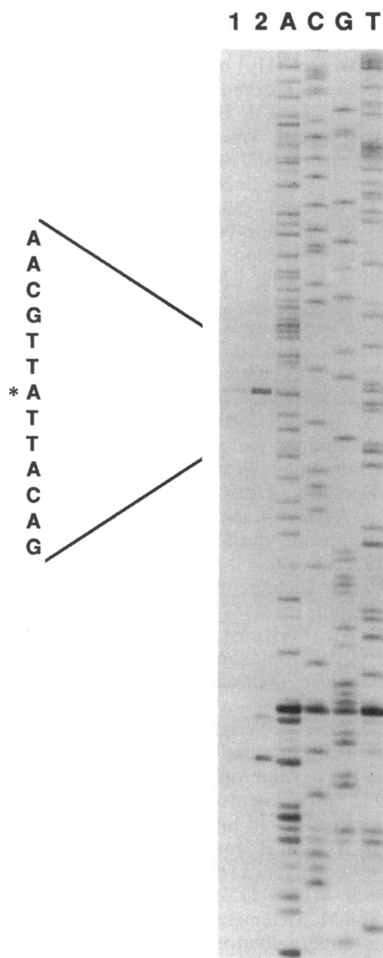

FIG. 5. Reverse transcriptase mapping of a transcription start site upstream from *sacX*. Extension was performed with primer AM2 (Fig. 4) and RNA extracted from CAM91 (lane 1) and CAM93 (lane 2). Extension products were labelled during elongation with [α-^{35}S]dATP and analyzed by electrophoresis along with DNA sequencing reactions performed with the same primer. The asterisk indicates the transcription start site.

constitute an operon whose transcription is subject to double control by the *degS-degU* regulatory system and by sucrose.

Both sucrose and stimulation by DegU are also required for maximal transcription of *sacB*. It has been suggested that null and hyperactive mutations affecting the DegU effector could mimic the absence and the presence, respectively, of a signal transmitted by the signalling system and that the concomitant presence of this hypothetical signal and external sucrose could represent the conditions under which the synthesis of levan by levansucrase is of value to the cell (29). The *degU32*(Hy) mutation and the presence of sucrose also cooperatively enhanced *sacY* transcription (fusion I; Fig. 3). This result suggests that the regulation exerted on this gene, encoding a positive regulator required for full expression of *sacB*, constitutes an additional lock on levansucrase synthesis under unsuitable conditions. The *sacXp3* (formerly *sacS^{h}3*) mutation, leading to overproduction of levansucrase, appears to create a relatively strong constitutive (sucrose-independent) promoter just upstream of *sacX*, resulting in a high level of transcription of *sacY* in the *degU*$^+$ background.

TABLE 3. Sucrose induction of *sacX* (visualized with fusion III) in *sacY* and *sacT* mutants

Recipient	Relevant genotype[a]	β-Galactosidase activity[b] with (+) or without (−) induction by sucrose	
		−	+
GM720		0.5	4.2
GM788	*sacXY*Δ*4*	0.6	3.9
GM820	*sacY::neo*	0.6	3.8
GM824	*sacT::neo*	0.4	2.4
GM836	*sacXY*Δ*4 sacT::neo*	0.3	0.2

[a] All strains carry the *degU32* mutation.
[b] In Miller units.

Stimulation by DegU and induction by sucrose of *sacXY* appear to act at different targets, as in the case of *sacB*. *cis*-acting sequences mediating regulation by DegU are located upstream of a σ^A-like promoter characterized in this work. Deletion analysis showed that full stimulation by DegU of the expression of a *sacX'-lacZ* fusion required sequences between −112 and −55 (Table 2). This observation is very similar to that of Henner et al. (7) for the activation of the *sacB* promoter (−117 and −96). A transcription start site was mapped 287 nt upstream of the *sacX* initiation codon; transcription initiated at the same point in the *degU::neo* and *degU32* mutants and, as expected, the steady-state level of the transcripts was higher in the *degU32* mutant (Fig. 5). Hence, it is likely that DegU (directly or indirectly) activates transcription from the *sacX* promoter.

Induction by sucrose does not affect initiation from the *sacX* promoter, as shown by the phenotype of fusion IV. A fusion derived from fusion I (Fig. 3) by deletion of the *Sal*I-*Nco*I fragment yielded similar results (data not shown). This result indicated that sequences negatively involved in sucrose induction of both *sacX* and *sacY* were located between +125 and the *sacX* initiation codon (+288) (Fig. 4). Antiterminators for levansucrase and endocellular sucrase expression, SacY and SacT, respectively (see above), were involved in this control (Table 3). This result strongly suggests that the target for induction is RNA. The *sacXp3* mutation renders transcription of the operon constitutive, presumably because the transcripts initiated from the new promoter do not contain the sequences involved in induction.

SacY, SacT, and BglG form a family of proteins that prevent termination, probably by interacting with homologous RAT sequences overlapping conditional terminators (see above). The *sacX* upstream region contains a sequence whose strong similarity with RAT sequences (Fig. 6) suggests that it might be involved as a binding site for SacY and SacT in induction of *sacXY* by sucrose. The RAT sequences are imperfectly palindromic (Fig. 6); a set of mutations in *sacB* RAT, including pairs of compensatory substitutions, indicated that a stem-loop structure in the mRNA was required for function (3). A similar mutational analysis indicated that the *sacX* leader RAT-like sequence was positively involved in sucrose induction of a *sacX-lacZ* fusion (31). However, the location of this sequence was somewhat surprising: (i) no obvious overlapping rho-independent terminator could be identified; and (ii) sequences at a distance of at least 100 nt downstream played a major role in induction of *sacXY* by sucrose. These sequences, playing a

+26 +54 +155

sacXY GGAUUGUGACUGGGCAGGCAGGCAAGACC.... // (*Sal*I)

sacB GGUUUGUUACUGAUAAAGCAGGCAAGACC

sacPA GGAUUGUGACUGGUAAAGCAGGCAAGACC

bgl- t1 GGAUUGUUACUGCAUUCGCAGGCAAAACC

FIG. 6. Putative target for anterminators in the *sacX* leader region. Shown is homology between a target for SacT and SacY in the *sacX* leader region and targets (RAT sequences) for SacY, SacT, and BglG in the leader regions of *sacB*, *sacPA*, and the *E. coli bgl* operon (t1 terminator), respectively. Nucleotides conserved in the four sequences are in boldface type. Inverted arrows indicate the terminator palindromes in the *sacB*, *sacPA*, and *bgl* leader regions. Broken inverted arrows indicate a putative terminator centered on the *Sal*I site (Fig. 4).

negative role, contain the 3′ portion of a palindrome whose structure is reminiscent of a transcription terminator (Fig. 6).

The results presented here do not allow the construction of a detailed model for induction by sucrose of *sacXY*; however, they provide evidence for a rather original mechanism, which might be antitermination, but with unusual features (the antiterminators acting at a great distance from the termination site) or another mechanism, in which SacY and SacT play a positive role by, for example, stabilizing the mRNA. These hypotheses are currently under investigation.

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REFERENCES

1. **Aymerich, S., G. Gonzy-Tréboul, and M. Steinmetz.** 1986. 5′-Noncoding region *sacR* is the target of all identified regulation affecting the levansucrase gene in *Bacillus subtilis*. J. Bacteriol. **166:**993–998.
2. **Aymerich, S., and M. Steinmetz.** 1987. Cloning and preliminary characterization of the *sacS* locus from *Bacillus subtilis* which controls the regulation of the exoenzyme levansucrase. Mol. Gen. Genet. **208:**114–120.
3. **Aymerich, S., and M. Steinmetz.** Submitted for publication.
4. **Brückner, R., O. Shoseyov, and R. H. Doi.** 1990. Multiple active forms of a novel serine protease from *Bacillus subtilis*. Mol. Gen. Genet. **221:**486–490.
5. **Crutz, A. M., M. Steinmetz, S. Aymerich, R. Richter, and D. Le Coq.** 1990. Induction of levansucrase in *Bacillus subtilis*: an antitermination mechanism negatively controlled by the phosphotransferase system. J. Bacteriol. **172:**1043–1050.
6. **Débarbouillé, M., M. Arnaud, A. Fouet, A. Klier, and G. Rapoport.** 1990. The *sacT* gene regulating the *sacPA* operon in *Bacillus subtilis* shares strong homology with transcriptional antiterminators. J. Bacteriol. **172:**3966–3973.
7. **Henner, D. J., M. Yang, L. Band, H. Shimotsu, M. Ruppen, and E. Ferrari.** 1986. Genes of *Bacillus subtilis* that regulate the expression of degradative enzymes, p. 81–90. *In* M. Alacevic, D. Hranueli, and Z. Toman (ed.), Genetics of industrial microorganisms. Proceedings of the Fifth International Symposium on the Genetics of Industrial Microorganisms. Pliva, Zagreb, Yugoslavia.
8. **Henner, D. J., M. Yang, and E. Ferrari.** 1988. Localization of *Bacillus subtilis sacU*(Hy) mutations to two linked genes with similarities to the conserved procaryotic family of two-component signalling systems. J. Bacteriol. **170:**5102–5109.
9. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150:**804–814.
10. **Houman, F., M. R. Diaz-Torres, and A. Wright.** 1990. Transcriptional antitermination in the *bgl* operon of *E. coli* is modulated by a specific RNA binding protein. Cell **62:**1153–1163.
11. **Itaya, M., K. Kondo, and T. Tanaka.** 1989. A neomycin resistance cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. Nucleic Acids Res. **17:**4410.
12. **Kunst, F., M. Debarbouillé, T. Msadek, M. Young, C. Mauel, D. Karamata, A. Klier, G. Rapoport, and R. Dedonder.** 1988. Deduced polypeptides encoded by the *Bacillus subtilis sacU* locus share homology with two-component sensor-regulator systems. J. Bacteriol. **170:**5093–5101.
13. **Le Coq, D., S. Aymerich, and M. Steinmetz.** 1991. Dual effect of a Tn*917* insertion into the *Bacillus subtilis sacX* gene. J. Gen. Microbiol. **137:**101–106.
14. **Le Coq, D., R. Richter, A. M. Crutz, and M. Steinmetz.** Unpublished data.
15. **Lepesant, J. A., F. Kunst, J. Lepesant-Kejzlarova, and R. Dedonder.** 1972. Chromosomal location of mutations affecting sucrose metabolism in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **118:**135–160.
16. **Mahadevan, S., and A. Wright.** 1987. A bacterial gene involved in transcription antitermination: regulation at a rho-independent terminator in the *bgl* operon of *E. coli*. Cell **50:**485–494.
17. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Morrisson, D. A., and B. Jaurin.** 1990. *Streptococcus pneumoniae* possesses canonical *Escherichia coli* (sigma 70) promoters. Mol. Microbiol. **4:**1143–1152.
19. **Podvin, L., and M. Steinmetz.** A *degU*-containing SPβ prophage complements superactivator mutations affecting the *Bacillus subtilis degSU* operon. Res. Microbiol., in press.
20. **Poth, H., and P. Youngman.** 1988. A new cloning system for *Bacillus subtilis* comprising elements of phage, plasmid and transposon vectors. Gene **73:**215–226.
21. **Rosenthal, R., P. A. Toye, R. Z. Korman, and S. A. Zahler.** 1979. The prophage of SPβ*c2dcitK-1*, a defective specialized transducing phage of *Bacillus subtilis*. Genetics **92:**721–739.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
23. **Schnetz, K., and B. Rak.** 1988. Regulation of the *bgl* operon of *Escherichia coli* by transcription antitermination. EMBO J. **7:**3271–3277.
24. **Shimotsu, H., and D. Henner.** 1986. Construction of a single copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. Gene **43:**85–94.
25. **Shimotsu, H., and D. J. Henner.** 1986. Modulation of *Bacillus subtilis* levansucrase gene expression by sucrose and regulation of the steady-state mRNA level by *sacU* and *sacQ* genes. J. Bacteriol. **168:**380–388.
26. **Sloma, A., A. Ally, D. Ally, and J. Pero.** 1988. Gene encoding a minor extracellular protease in *Bacillus subtilis*. J. Bacteriol. **170:**5557–5563.
27. **Steinmetz, M.** Unpublished data.
28. **Steinmetz, M., and S. Aymerich.** 1986. Analyse génétique de *sacR*, régulateur en *cis* de la synthèse de la lévane-saccharase de *Bacillus subtilis*. Ann. Microbiol. (Paris) **137A:**3–14.
29. **Steinmetz, M., and S. Aymerich.** 1990. The *Bacillus subtilis sac-deg* constellation: how and why? p. 303–311. *In* M. M. Zukowski, A. T. Ganesan, and J. A. Hoch (ed.), Genetics and biotechnology of bacilli, vol. 3. Academic Press, Inc., New York.
30. **Steinmetz, M., D. Le Coq, S. Aymerich, G. Gonzy-Tréboul, and**

P. Gay. 1985. The DNA sequence of the gene for the secreted *Bacillus subtilis* enzyme levansucrase and its genetic control sites. Mol. Gen. Genet. **200:**220–228.

31. **Tortosa, P., M. Steinmetz, and D. Le Coq.** Unpublished data.
32. **Vandeyar, M. A., and S. A. Zahler.** 1986. Chromosomal insertions of Tn*917* in *Bacillus subtilis*. J. Bacteriol. **167:**530–534.
33. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
34. **Youngman, P.** 1990. Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus* species, p. 221–257. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley and Sons Ltd., Chichester, England.
35. **Zagorec, M., and M. Steinmetz.** 1990. Expression of levansucrase-β-galactosidase hybrids inhibits secretion and is lethal in *Bacillus subtilis*. J. Gen. Microbiol. **136:**1137–1143.
36. **Zagorec, M., and M. Steinmetz.** 1991. Construction of a derivative of Tn*917* containing an outward directed promoter and its use in *Bacillus subtilis*. J. Gen. Microbiol. **137:**107–112.
37. **Zahler, S. A., R. Z. Korman, J. M. Odebralski, P. S. Fink, C. J. Mackey, C. G. Poutré, R. Lipsky, and P. J. Youngman.** 1982. Genetic manipulations with phage SPβ, p. 41–50. *In* A. T. Ganesan, S. Chang, and J. A. Hoch (ed.), Molecular cloning and gene regulation in bacilli. Academic Press, Inc., New York.
38. **Zukowski, M. M., L. Miller, P. Cogswell, K. Chen, S. Aymerich, and M. Steinmetz.** 1990. Nucleotide sequence of the *sacS* locus of *Bacillus subtilis* reveals the presence of two regulatory genes. Gene **90:**153–155.