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Regulation of the *sacPA* Operon of *Bacillus subtilis*: Identification of Phosphotransferase System Components Involved in SacT Activity

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The *sacT* gene which controls the *sacPA* operon of *Bacillus subtilis* encodes a polypeptide homologous to the *B. subtilis* SacY and the *Escherichia coli* BglG antiterminators. Expression of the *sacT* gene is shown to be constitutive. The DNA sequence upstream from *sacP* contains a palindromic sequence which functions as a transcriptional terminator. We have previously proposed that SacT acts as a transcriptional antiterminator, allowing transcription of the *sacPA* operon. In strains containing mutations inactivating *ptsH* or *ptsI*, the expression of *sacPA* and *sacB* is constitutive. In this work, we show that this constitutivity is due to a fully active SacY antiterminator. In the wild-type *sacT*$^+$ strain or in the *sacT30* mutant, SacT requires both enzyme I and HPr of the phosphotransferase system (PTS) for antitermination. It appears that the PTS exerts different effects on the *sacB* gene and the *sacPA* operon. The general proteins of the PTS are not required for the activity of SacY while they are necessary for SacT activity.

In *Bacillus subtilis*, the expression of both the *sacPA* operon and the *sacB* gene are induced by sucrose (21). The *sacA* gene codes for an endocellular sucrase (10, 11, 22), and *sacP* is the structural gene of a membrane-associated, specific component of the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS) for sucrose transport (8, 9). The *sacB* gene encodes a second sucrose-hydrolyzing enzyme called levansucrase (22, 37). Two regulatory loci, *sacT* and *sacS*, control the transcription of these genes (21, 22). Transcriptional regulation of the *sacB* gene involves an antitermination mechanism (31, 35): between the promoter and the *sacB* coding sequence, a region of dyad symmetry acts as a transcriptional terminator. Deletion of this termination structure or single-base changes that modify the dyad symmetry led to constitutive synthesis of levansucrase (34). The regulatory locus *sacS* was identified by analysis of several constitutive mutants. It contains two genes, *sacX* and *sacY*, which seem to form an operon. The *sacY* gene encodes an antiterminator similar to the *bglG* gene product of *Escherichia coli* (2, 30, 42). The *sacX* gene encodes an enzyme II-like protein, similar to *sacP* of *B. subtilis*, *bglF* from *E. coli*, and *scrA* from *E. coli*, *Streptococcus mutans*, and *Vibrio alginolyticus* (3, 43). A specific component of the PTS is also involved in induction of the *bgl* operon of *E. coli*. A model of regulation of the *bgl* operon has been proposed: the enzyme II^{Bgl}, which is involved in β-glucoside transport, negatively regulates the positive regulator BglG by phosphorylation, and thereby abolishing its activity (1, 29).

A similar model of regulation was proposed for *sacB*. In the absence of inducer, the *sacB* gene is not transcribed. SacX might be phosphorylated by enzyme I via HPr and an enzyme III and could then inhibit the antiterminator SacY by phosphorylation. In the presence of sucrose, SacX and SacY would be fully dephosphorylated and thereby activated (2, 4).

Genetic and biochemical studies have shown that *sacP* mutants do not take up sucrose (9, 22). It seems, therefore, that the *sacX* gene product is not involved in sucrose uptake in the wild type. This suggests that SacX is a minor but nevertheless functional enzyme II^{Scr} playing a regulatory role (4).

The *sacT* locus regulating the expression of the *sacPA* operon has been identified by a mutation called *sacT30*, leading to the constitutive expression of *sacPA* (21). The *sacT* gene located upstream of the *sacPA* operon encodes a polypeptide homologous to the *B. subtilis* SacY and *E. coli* BglG antiterminators (5). The DNA sequence upstream of the *sacP* gene contains a palindromic sequence which is similar to a transcriptional termination site and almost identical to that found upstream of *sacB*. Thus, we have proposed that SacT acts as a transcriptional antiterminator, allowing the RNA polymerase to pass through a terminator located between the *sacPA* promoter and *sacP*. "Cross talk" between the two regulatory systems controlling the *sacPA* and *sacB* genes has been observed. A constitutive level of *sacB* expression is detected in the *sacT30* mutant. Conversely, in *sacY*(Con) mutants, the *sacPA* operon expression is constitutive (22, 36). In a strain containing a deletion of *ptsH*, *ptsI*, and *ptsG* genes encoding HPr, enzyme I, and enzyme II^{Glc}, respectively, the expression of *sacPA* and *sacB* genes is also constitutive (4, 5). This result strongly suggests that the PTS negatively regulates the SacT and SacY activities by phosphorylation.

In this study, we have reinvestigated the role of SacT in *sacPA* regulation in a variety of mutants, including PTS mutants (5, 21, 22). The results presented here indicate that both in the wild-type strain grown in the presence of sucrose and in the constitutive *sacT30* mutant strain, SacT needs both enzyme I and HPr to be an active antiterminator. It was also shown that the weak constitutive expression of *sacPA*

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TABLE 1. *B. subtilis* strains used in this study[a]

Strain	Genotype or description	Source[b] or reference
168	*trpC2*	Laboratory stock
GM329	*trpC2 metC3 ptsGHI::erm*[c]	7
QB39	*sacT30*	Laboratory stock
PG585	*trpC2 metC3 ptsI7*[d]	Laboratory stock
MZ303	*ptsH::cat*[e]	This work
QB934	*trpC2 metC3 glyB133 tre-12*	Laboratory stock
QB4383	*trpC2 sacXY::aphA3*[f]	pMF10→168
QB4508	*sacT30 sacP::cat*[g]	pBSG8-15→QB39
QB6001	*trpC2 sacT::aphA3*[h]	pTP3→168
QB6010	*trpC2 amyE::(sacP'-lacZ cat)*	pTP7 tf→168
QB6011	*sacT30 amyE::(sacP'-lacZ cat)*	pTP7 tf→QB39
QB6014	*trpC2 ptsGHI::erm*[c] *amyE::(sacP'-lacZ cat)*	GM329 tf→QB6010
QB6021	*trpC2 ptsGHI::erm*[c] *sacXY::aphA3*[f]	GM329 tf→QB4383
QB6022	*trpC2 ptsGHI::erm*[c] *sacT::aphA3*[h]	GM329 tf→QB6001
QB6023	*trpC2 sacXY::aphA3*[f] *amyE::(sacP'-lacZ cat)*	pTP7 tf→QB4383
QB6024	*trpC2 sacT::aphA3*[h] *amyE::(sacP'-lacZ cat)*	pTP7 tf→QB6001
QB6026	*trpC2 ptsGHI::erm*[c] *sacXY::aphA3*[f] *amyE::(sacP'-lacZ cat)*	pTP7 tf→QB6021
QB6027	*trpC2 ptsGHI::erm*[c] *sacT::aphA3*[h] *amyE::(sacP'-lacZ cat)*	pTP7 tf→QB6022
QB6039	*sacT30 ptsGHI::erm*[c] *amyE::(sacP'-lacZ cat)*	GM329 tf→QB6011
QB6040	*sacT30 sacXY::aphA3*[f] *amyE::(sacP'-lacZ cat)*	QB4383 tf→QB6011
QB6041	*sacT30 ptsGHI::erm*[c] *sacXY::aphA3*[f] *amyE::(sacP'-lacZ cat)*	GM329 tf→QB6040
QB6042	*sacT30 amyE::(sacP'-lacZ aphA3)*	pTP11 tf→QB39
QB6044	*sacT30 sacP::cat*[g] *amyE::(sacP'-lacZ aphA3)*	pTP11 tf→QB4508
QB6046	*sacT30 ptsG::cat*[i] *amyE::(sacP'-lacZ aphA3)*	pJR100 tf→QB6042
QB6047	*sacT30 ptsH::cat*[e] *amyE::(sacP'-lacZ aphA3)*	MZ303 tf→QB6042
QB6048	*trpC2 sacXY::aphA3*[f] *sacXY*::pMF22	pMF22 tf→QB4383
QB6049	*trpC2 sacXY::tet*[f]	QB6048 tf→168
QB6050	*sacT30 ptsH::cat*[e] *sacXY::tet*[f] *amyE::(sacP'-lacZ aphA3)*	QB6049 tf→QB6047
QB6051	*sacT30 metC3 glyB133 trpC2 tre-12 amyE::(sacP'-lacZ aphA3)*	QB6042 tf→QB934
QB6052	*sacT30 sacXY::tet*[f] *metC3 glyB133 trpC2 tre-12 amyE::(sacP'-lacZ aphA3)*	QB6049 tf→QB6051
QB6053	*sacT30 sacXY::tet*[f] *metC3 glyB133 trpC2 tre-12 ptsI7*[d] *amyE::(sacP'-lacZ aphA3)*	QB6070 td→QB6052
QB6054	*trpC2 amyE::(sacP'-lacZ cat) sacPt2*	pBS G8-35 tf→168
QB6055	*trpC2 amyE::(sacP'-lacZ cat)*	pBS G8-38 tf→168
QB6056	*trpC2 amyE::(sacP'-lacZ cat) sacT::aphA3*[h] *sacPt2*	pBS G8-35 tf→QB6001
QB6057	*trpC2 amyE::(sacP'-lacZ cat) sacT::aphA3*[h]	pBS G8-38 tf→QB6001
QB6058	*trpC2 amyE::(sacP'-lacZ cat) sacPt1*	pBS G8-34 tf→168
QB6060	*trpC2 sacXY::aphA3*[f] *amyE::(sacP'-lacZ cat) sacPt2*	QB4383 tf→QB6054
QB6063	*trpC2 ptsGHI::erm*[c] *sacXY::aphA3*[f] *amyE::(sacP'-lacZ cat) sacPt2*	GM329 tf→QB6060
QB6064	*sacT30 sacP::cat*[g] *sacXY::tet*[f] *amyE::(sacP'-lacZ aphA3)*	QB6049 tf→QB6044
QB6065	*sacT30 ptsG::cat*[i] *sacXY::tet*[f] *amyE::(sacP'-lacZ aphA3)*	QB6049 tf→QB6046
QB6066	*trpC2 sacT::(sacT'-lacZ cat)*	pTZ2 tf→168
QB6067	*trpC2 sacT::aphA3*[h] *sacT::(sacT'-lacZ cat)*	QB6001 tf→QB6066
QB6068	*trpC2 ptsGHI::erm*[c] *sacT::(sacT'-lacZ cat)*	GM329 tf→QB6066
QB6069	*trpC2 sacXY::tet*[f] *sacT::(sacT'-lacZ cat)*	QB6049 tf→QB6066
QB6070	*trpC2 ptsI7*[d]	168 td→PG585

[a] *erm* is the erythromycin resistance gene of pHV1209 (7). *cat* is the pC194 chloramphenicol acetyltransferase gene (16) and *aphA3* is the *Streptococcus faecalis* kanamycin resistance gene. *tet* is the tetracycline resistance gene of the plasmid pBC16 of *Bacillus cereus* (17).

[b] tf → indicates transformation; td → indicates transduction.

[c] *pts::erm* corresponds to an insertion of the *erm* gene between the *ptsG* 3′ end and the *ptsI* 5′ end (7).

[d] *ptsI7* is a point mutation in the *ptsI* gene (13).

[e] *ptsH::cat* corresponds to the deletion of a 155-bp *Hin*dIII-*Hpa*I fragment within the *ptsH* gene which was replaced by the *cat* gene of pC194. The ligation of the *Stu*I site of *cat* to the *Hpa*I site of *ptsH* led to a fusion between *cat* and the end of *ptsH*, allowing expression of *ptsI*.

[f] *sacXY::tet* and *sacXY::aphA3* are insertions of *tet* or *aphA3* genes, respectively, between the *sacX* 3′ end and the *sacY* 5′ end (described in the text).

[g] *sacP::cat*, the internal *Eco*RV fragment of the *sacP* gene is replaced by the *cat* gene (5).

[h] *sacT::aphA3*, the *aphA3* gene was substituted to a *Bgl*II internal fragment of the *sacT* gene (5).

[i] *ptsG::cat* (a gift from J. Reizer), an insertion of the *cat* gene in the *ptsG* gene replaced in an internal fragment of about 400 bp (38).

observed for a *ptsGHI* null mutant was due to a fully active SacY.

MATERIALS AND METHODS

Media. *E. coli* strains were grown in Luria-Bertani (LB) broth, and *B. subtilis* strains were grown in Penassay antibiotic medium 3 (Difco), SP medium (8 g of nutrient broth per liter–1 mM $MgSO_4$–13 mM KCl, supplemented after sterilization with 2.5 μM $FeSO_4$, 500 μM $CaCl_2$, and 10 μM $MnCl_2$), or C medium [70 mM $K_2H\ PO_4 \cdot 3H_2O$, 30 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 0.5 mM $MgSO_4$, 0.01 mM $MnSO_4$, 22 mg of ferric ammonium citrate per liter]. C medium was supplemented with the following nutrients: 20 mM potassium succinate, 50 mM potassium glutamate, and 100 mg of auxotrophic requirements per liter (CSK medium).

Strains. All the strains used in this study are listed in Table 1. Strain MZ303 contains an internal *ptsH* deletion, allowing the expression of *ptsI*. A 155-bp *Hin*dIII-*Hpa*I restriction fragment (15) was replaced by the *cat* gene. In this strain, the

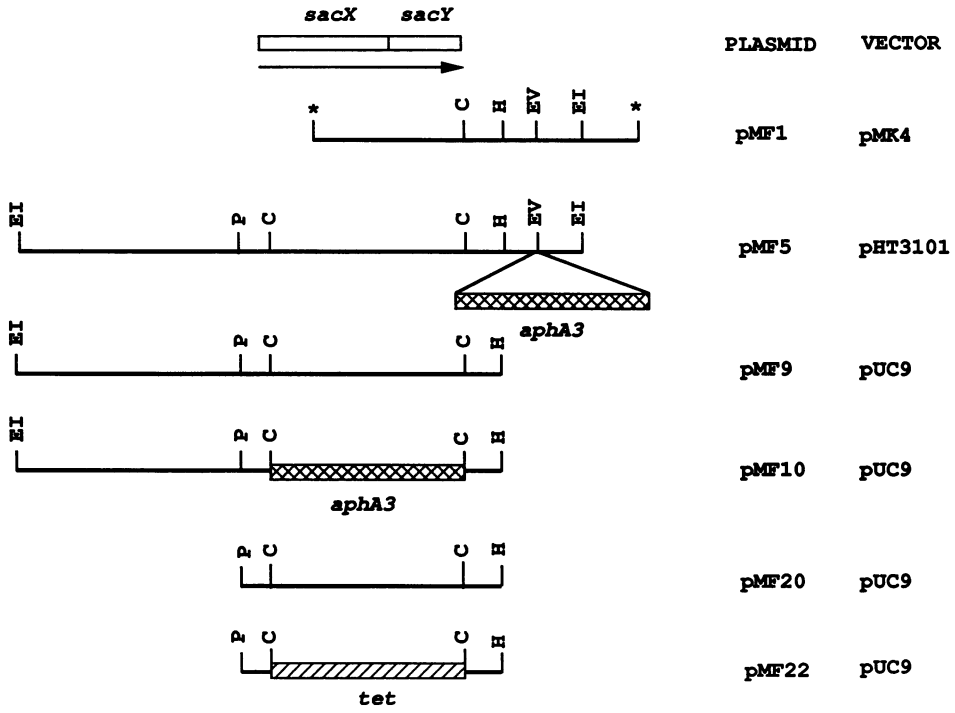

FIG. 1. Restriction maps of plasmids containing the *sacX* and *sacY* genes used in this study. Stars indicate the *Sau*3A restriction sites used during the construction of pMF1.

ptsH mutation has a nonpolar effect on the expression of the downstream *ptsI* gene. Indeed, after transduction of MZ303 strain with a recombinant SPβ prophage carrying *ptsG* and *ptsH* but not *ptsI*, the growth of the resulting transductant was restored on PTS sugars (Glc, Fru, Scr, Mtl, Man).

Plasmids. The pTP1 plasmid can replicate in both *E. coli* and *B. subtilis*. It contains the *sacT* gene and has previously been described (5). The pBSG8-27 plasmid was constructed as follows: a *Hin*dIII-*Stu*I DNA fragment containing the first 200 bp of *sacP* and 851 bp upstream from *sacP* was cloned between the *Hin*dIII and *Sma*I sites of pUC19 (41). The pJR100 plasmid (a gift from J. Reizer) contains the *ptsG* gene interrupted by a *cat* gene (see Fig. 4). The plasmids pMF10 and pMF22 (Fig. 1) were constructed as follows: the complete *sacX sacY* operon was cloned, starting with pMF1 (6), which contains the *sacY* gene of *B. subtilis* and part of the *sacX* gene. A 1.8-kb *Cla*I restriction fragment containing the *aphA3* gene conferring kanamycin resistance (39) was inserted at the unique *Eco*RV restriction site downstream from *sacY* in pMF1 (Fig. 1). The resulting recombinant plasmid was linearized, and the *aphA3* gene was integrated by a double crossover event into the chromosome of *B. subtilis* 168. An 11-kb *Eco*RI DNA fragment containing the *sacX sacY* operon and the *aphA3* gene was cloned in the *Eco*RI restriction site of pHT3101 (23), resulting in pMF5. A 7.5-kb *Eco*RI-*Hin*dIII fragment containing the *sacX sacY* operon was purified from pMF5 and cloned between the corresponding restriction sites of pUC9, giving pMF9. The inserted DNA fragment in pMF9 contains a *Cla*I restriction site in the middle of *sacX* and one downstream from *sacY* (35, 43). The pMF9 plasmid was restricted with *Cla*I, and the *Cla*I fragment was replaced by a *Cla*I DNA fragment containing the *aphA3* gene, resulting in pMF10. A 4.5-kb *Pst*I-*Hin*dIII restriction fragment overlapping the *sacX sacY* operon was cloned in the corresponding sites of pUC9 to give pMF20. A 2.5-kb *Ssp*I fragment containing the tetracycline resistance gene from pBC16 (17) was cloned between the *Cla*I sites of pMF20 to give pMF22. The *lacZ* translational fusions were constructed either in two steps by using the pIS112 (24) and pAF1 vectors (12) or in a single step by using pAC2 (Cm^r) (25) or pAC7 (Km^r) (40) (Fig. 2). All the resulting plasmids carrying translational *lacZ* fusions are derivatives of ptrpBG1 (32), allowing single-copy integration at the *amyE* locus via homologous recombination. The plasmid pBSG8-32 and its derivatives were constructed as follows: the 1-kb DNA fragment from pBSG8-27 flanked by a *Hin*dIII site, and the *Eco*RI site of the polylinker was cloned between the corresponding sites of mp18 replicative form DNA. A single-strand M13 recombinant phage, called ϕ1, was obtained and used for site-directed mutagenesis (see below). A derivative of ϕ1, called ϕ2, contains a single-base change in the transcriptional terminator of the *sacPA* operon (Fig. 3B). The replicative form of the ϕ2 phage was purified, and the *Xmn*I-*Ssp*I DNA fragment containing the promoter, the modified transcriptional terminator, and the beginning of the *sacP* coding sequence was cloned at the *Bam*HI site (made blunt) of the *lacZ* gene of pIS112. The resulting plasmid containing an in-frame translational fusion was called pBSG8-32. The translational fusion was then transferred to pAF1, which contains a promoterless *lacZ* gene and two fragments of the *B. subtilis amyE* gene. The pAF1 plasmid also contains the *cat* gene, allowing selection of integrants. The *cat* and *lacZ* genes of pIS112 contain unique *Stu*I and *Sac*I restriction sites. An *Stu*I-*Sac*I DNA fragment containing the translational fusion was inserted between the *Stu*I and *Sac*I restriction sites of pAF1 to give plasmid pBSG8-34. The corresponding wild-type *Xmn*I-*Ssp*I DNA fragment of pBSG8-27 was similarly cloned in pIS112 to give pBSG8-37 and transferred into pAF1 to give plasmid pBSG8-38. The pBSG8-35 plasmid was constructed as follows. pBSG8-34

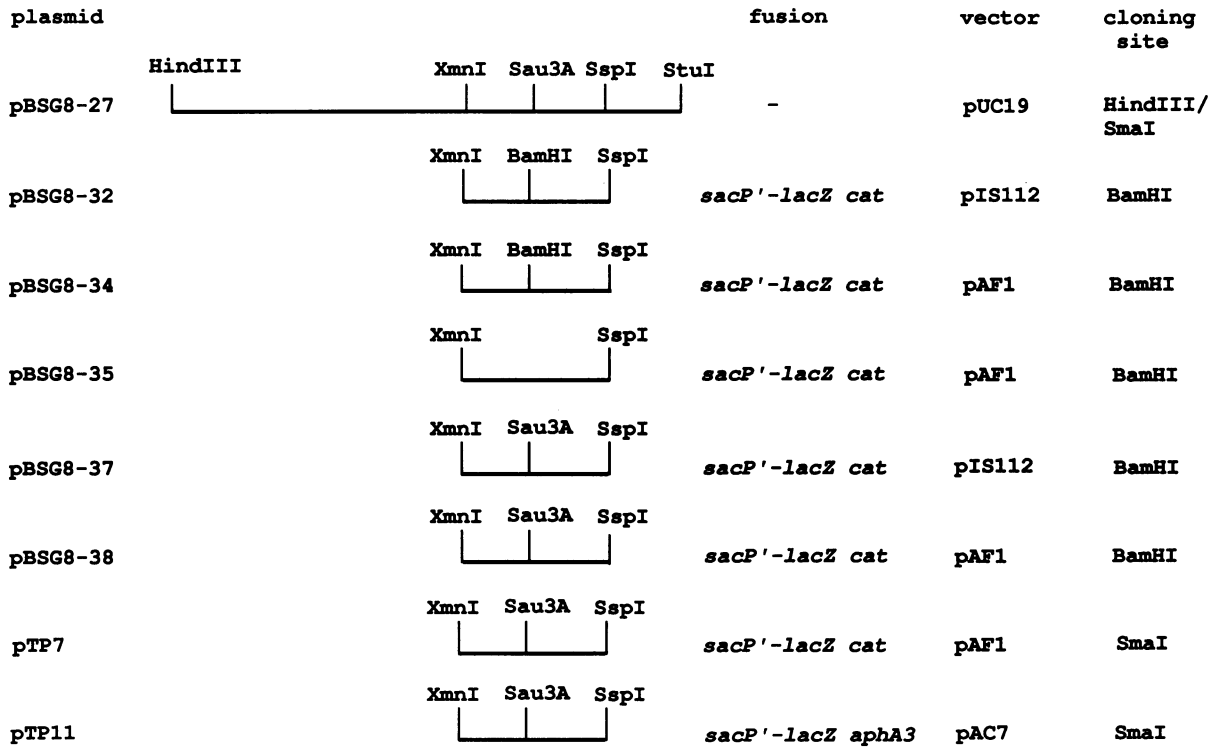

FIG. 2. Construction of *sacP-lacZ* fusions. Translational fusions constructed in pIS112 were transferred into pAF1 as described in Materials and Methods.

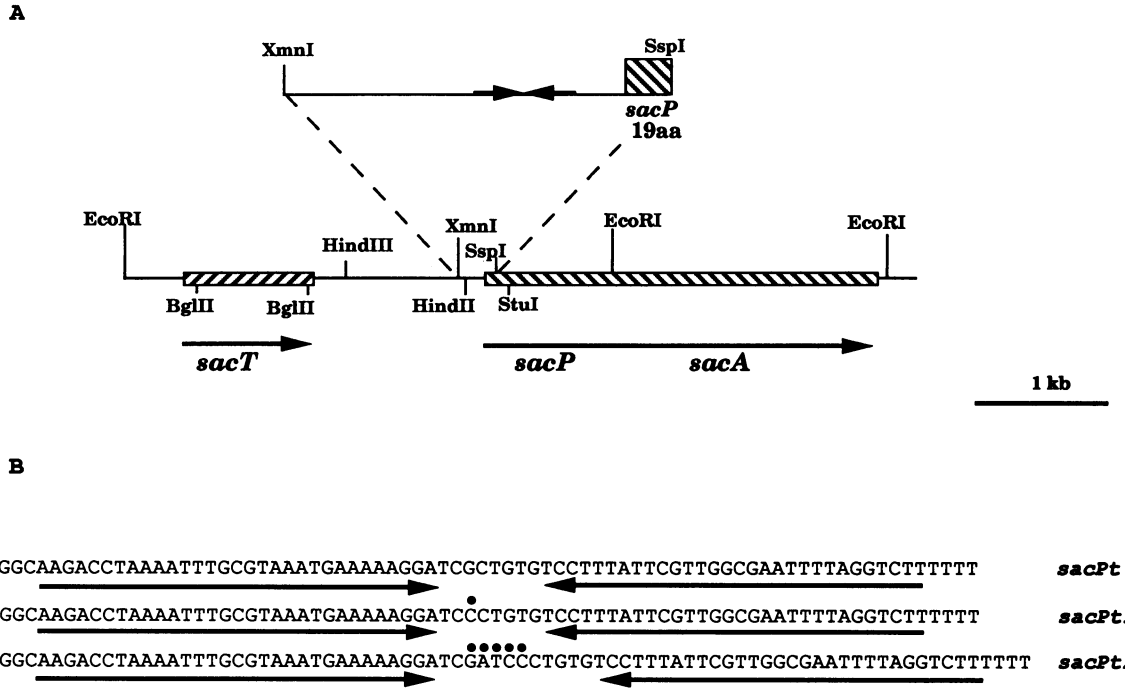

FIG. 3. (A) Simplified restriction map of the *sacTPA* region of *B. subtilis*. The upper line indicates the position of the *Xmn*I-*Ssp*I restriction fragment (300 bp) which contains the promoter. Convergent arrows indicate the palindromic sequence. (B) Mutations created in vitro in the palindromic sequence. Dots indicate the location of mutations. In *sacPt1*, the introduction of a C in the place of a G creates a *Bam*HI restriction site. In *sacPt2*, the *Bam*HI restriction site was made blunt by using Klenow fragment.

TABLE 2. Effects on the expression of *sacP-lacZ* fusion of mutations introduced into the palindromic structure[a]

Strain	Relevant genotype	Palindromic structure	β-Galactosidase activity (Miller units/mg of protein) with:		
			No sugar added	0.15% sucrose	0.15% sucrose and 0.1% glucose
QB6055	*sacT*$^+$ *sacP-lacZ*	WT	38	940	150
QB6057	Δ*sacT sacP-lacZ*	WT	27	27	7
QB6058	*sacT*$^+$ *sacP-lacZ*	t1	16	650	
QB6054	*sacT*$^+$ *sacP-lacZ*	t2	400	800	90
QB6056	Δ*sacT sacP-lacZ*	t2	395	382	37

[a] Cells were grown on CSK medium with or without glucose or sucrose, as indicated. WT, wild type; t1 and t2, mutated palindromic structures (see Fig. 3B).

was restricted by using *Bam*HI and treated with the Klenow fragment of DNA polymerase I in the presence of the four dXTPs. The DNA was religated to give plasmid pBSG8-35. As a result, four bases were added in the loop of the palindromic sequence located upstream from the *sacP* gene. The pTP7 plasmid has previously been described (5): the *Xmn*I-*Ssp*I restriction fragment of pBG8-27 was cloned in the *Sma*I site of the *lacZ* gene of pIS112, and the fusion was transferred into pAF1 to give pTP7. The pTP11 plasmid contains the same translational fusion as pTP7, but the *cat* gene is replaced by an *aphA3* gene. The pTP11 plasmid contains the same *Xmn*I-*Ssp*I restriction fragment but cloned in the pAC7 vector. A 716-bp *Eco*RI-*Eco*RV DNA fragment containing the beginning of *sacT* was cloned between the *Eco*RI and *Sma*I sites of pAC2 to give pTZ2. In pTZ2, 73 codons of *sacT* were cloned in frame with *lacZ*.

Site-directed mutagenesis. The Muta-Gene M13 kit (Bio-Rad Laboratories, Richmond, Calif.) was used for site-directed mutagenesis. The sequence of the 45-mer oligonucleotide used as the primer was 5′-AGG ACA CAG GGA TTC TTT TTC ATT TAC GCA AAT TTT AGG TCT TGC-3′. The underlined G replaces a C in the wild-type sequence. The presence of the mutation in the M13 phage was confirmed by sequencing single-strand phage DNA. Sequencing was performed according to the method of Sanger et al. by using a series of oligonucleotides priming at intervals of 200 nucleotides (28).

Transformation of *B. subtilis*. Transformation was performed as previously described (18). Antibiotic-resistant transformants were selected on SP medium containing chloramphenicol (5 μg/ml), tetracycline (20 μg/ml), and kanamycin (5 μg/ml). To select erythromycin-resistant transformants, erythromycin (1 μg/ml) and lincomycin (25 μg/ml) were added to SP medium.

β-Galactosidase assays. *B. subtilis* cells containing *lacZ* fusions were grown in CSK medium supplemented with 0.1% glucose or 0.15% sucrose as indicated in the tables. β-Galactosidase assays were carried out as previously described (26).

RESULTS

Alteration of the palindromic sequence upstream from *sacPA*. In previous work (5), we proposed that SacT functions as an antiterminator and interacts with the palindromic sequence located between the promoter and the *sacP* gene (Fig. 3A). A constitutive mutant in which *sacPA* is expressed in the absence of sucrose has previously been isolated (21): the constitutive *sacT30* mutation corresponds to an Asp-96 to Tyr missense mutation located in a region highly conserved between SacT and SacY (5). Since no mutation in the putative termination structure had been identified, we decided to test the hypothesis that the palindromic sequence functions as a transcriptional terminator. Two mutations were created in vitro to modify this region of dyad symmetry (Fig. 3B). A single-base change was introduced by site-directed mutagenesis between the two inverted repeats (see Materials and Methods). This mutation creates a *Bam*HI restriction site, which was first introduced upstream from a *sacP-lacZ* translational fusion and then reintroduced as a single copy at the *amyE* locus of the *B. subtilis* chromosome, giving strain QB6058 (*sacPt1*). By using plasmid pBSG8-34 containing the *Bam*HI restriction site, four nucleotides were also inserted by using a Klenow fragment. This mutation was introduced upstream from the *sacP-lacZ* fusion in plasmid pBSG8-35, allowing integration into the chromosome by a double crossover event at the *amyE* locus, resulting in strain QB6054 (*sacPt2*). The expression of *lacZ* was assayed for these different strains (which do or do not contain a wild-type allele of the *sacT* gene) with and without induction by sucrose (Table 2). The single-base change creating the *Bam*HI restriction site in strain QB6058 had a small effect on the expression of the *sacPA* operon. However, the introduction of four bases in the middle of the palindrome resulted in weak constitutive expression of the *sacPA* operon (for example, in strain QB6054). This result is surprising, since the calculated ΔG of the secondary structure is not greatly modified by these mutations. It is therefore likely that this modification affects the target of the SacT regulatory protein. Addition of sucrose to the growth medium resulted in a twofold increase in *lacZ* expression in strain QB6054 (*sacPt2*). This result indicates that overinduction by sucrose is dependent on the presence of SacT.

TABLE 3. Regulation of *sacT* gene expression in *B. subtilis*[a]

Strain	Relevant genotype	β-Galactosidase activity (Miller units/mg of protein) with:		
		No sugar added	0.15% sucrose	0.1% glucose
QB6066	*sacT-lacZ*	70	96	83
QB6067	*sacT-lacZ* Δ*sacT*	70		
QB6068	*sacT-lacZ* Δ*pts*(*GHI*)	73		
QB6069	*sacT-lacZ* Δ(*sacX sacY*)	76		

[a] Cells were grown on CSK medium with or without glucose or sucrose, as indicated.

The induced level of expression of β-galactosidase in strain QB6055 (*sacT*$^+$ *sacP-lacZ*) which has a wild-type terminator was sixfold lower when glucose was present in the growth medium. It has already been shown that the synthesis of sucrase is markedly repressed by various carbon sources, including glucose (19, 22). This result indicates that in strain QB6055, the *Xmn*I-*Ssp*I DNA fragment containing the inducible promoter of the *sacPA* operon also includes the target involved in the glucose effect. In strain QB6056 (Δ*sacT*) grown in the presence of glucose, a 10-fold reduction of *lacZ* expression was also observed. This result suggests that the glucose effect requires the presence of the *Xmn*I-*Ssp*I fragment in the promoter region of the *sacPA* operon and is SacT independent.

Control of *sacT* gene expression. The regulation of *sacT*

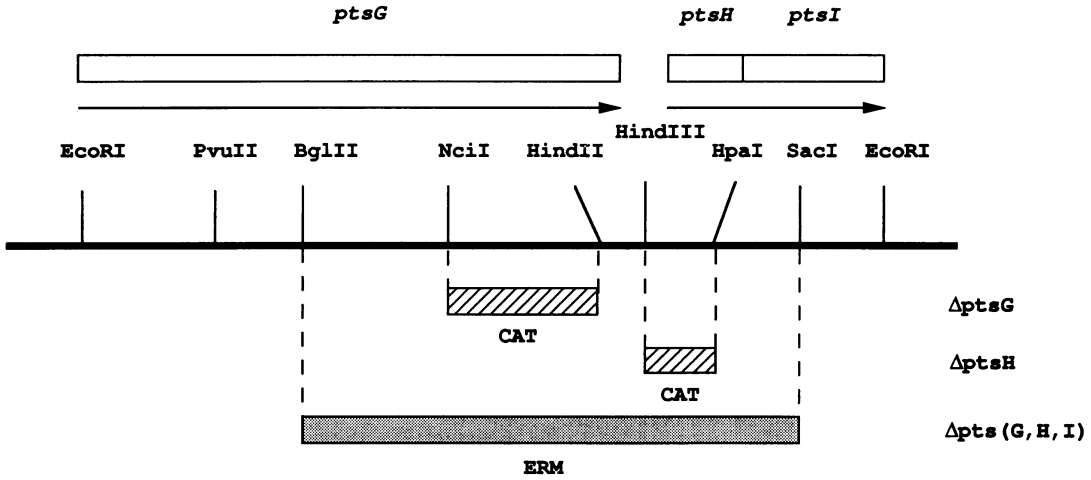

FIG. 4. Restriction map of the *ptsGHI* region of *B. subtilis*. In the Δ*pts*(GHI) strain, the *Bgl*II-*Sac*I fragment was substituted with the *ermC* gene (15). In the Δ*ptsG* strain, the *cat* gene was cloned between the *Nci*I-*Hin*dII fragment of the *ptsG* gene. In the Δ*ptsH* strain, the *cat* gene was inserted between the *Hin*dIII and *Hpa*I sites of *ptsH*. A translational fusion was made between *cat* and the end of *ptsH*, allowing the expression of *ptsI* (not shown).

gene expression was analyzed by using gene fusions. In order to construct a *sacT-lacZ* translational fusion, an *Eco*RI-*Eco*RV DNA fragment, containing the beginning of the *sacT* coding sequence and the 500 bp upstream from the ATG initiation codon, was cloned in pAC2 (see Materials and Methods). The resulting plasmid, called pTZ2, was introduced by transformation into *B. subtilis* 168, and Erm^r colonies were isolated. Since the promoter of the *sacT* gene was not localized, only fusions integrated by a single crossover event at the *sacT* locus were retained, and one such strain was reisolated. In this strain, QB6066, the entire plasmid was integrated into the chromosome by a Campbell-type recombination event via the *Eco*RI-*Eco*RV homologous DNA fragment. The *sacT-lacZ* fusion was mapped by transformation at the *sacT* locus. Therefore, the *sacT-lacZ* fusion is expressed from the chromosomal promoter of the *sacT* gene. β-Galactosidase in cultures of QB6066 (*sacT-lacZ*) grown in minimal medium containing sucrose or glucose as carbon sources was assayed (Table 3). The expression of *sacT* was neither induced by sucrose nor repressed by glucose in the culture medium. The replicative plasmid, pTP1, containing the entire *sacT* gene, was introduced by transformation into strain QB6066, and β-galactosidase in the transformants was assayed. The presence of pTP1 did not modify the expression of the *sacT-lacZ* fusion (not shown). It seems, therefore, that *sacT* is not negatively autoregulated. Two deletions, inactivating the *sacT* or the *sacX* and *sacY* genes, were introduced into strain QB6066, resulting in strains QB6067 and QB6069, respectively (Table 3). Assays of *lacZ* expression indicate that *sacT* gene expression is not negatively controlled by SacT or SacY. A large deletion encompassing *ptsH* encoding HPr, *ptsI* encoding enzyme I, and *ptsG* encoding enzyme II^{Glc} (Fig. 4) was introduced into QB6066, giving strain QB6068. The *sacT* gene expression did not appear to be controlled by the PTS in strain QB6068, as measured by *lacZ* expression (Table 3).

TABLE 4. Control of *sacP-lacZ* expression in *sacT*, *sacX*, *sacY*, and *pts* mutants[a]

Strain	Relevant genotype	β-Galactosidase activity (Miller units/mg of protein) with:	
		No sugar added	0.15% sucrose
QB6010	*sacP-lacZ*	13	620
QB6024	*sacP-lacZ* Δ*sacT*	36	14
QB6023	*sacP-lacZ* Δ(*sacX sacY*)	16	576
QB6014	*sacP-lacZ* Δ*pts*(*GHI*)	1,540	
QB6027	*sacP-lacZ* Δ*pts*(*GHI*) Δ*sacT*	1,660	
QB6026	*sacP-lacZ* Δ*pts*(*GHI*) Δ(*sacX sacY*)	15	
QB6011	*sacP-lacZ sacT30*	7,600	
QB6039	*sacP-lacZ sacT30* Δ*pts*(*GHI*)	1,610	
QB6040	*sacP-lacZ sacT30* Δ(*sacX sacY*)	7,800	
QB6041	*sacP-lacZ sacT30* Δ(*sacX sacY*) Δ*pts*(*GHI*)	24	

[a] Cells were grown on CSK medium with or without glucose or sucrose, as indicated.

Role of the PTS in the expression of the *sacPA* operon. It was previously shown that the PTS regulates the expression of the *sacPA* and *sacB* genes in *B. subtilis*. As proposed by Crutz et al. (4), SacX might be a sucrose sensor which is phosphorylated via the PTS and in this state inhibits the SacY antiterminator by transferring the phosphoryl group to SacY. Exogenous sucrose would lead to the dephosphorylation of SacX and consequently SacY, thereby allowing antitermination. It was suggested that SacT could be regulated by phosphorylation via the PTS (5). Thus, we constructed a series of strains in which the *sacP-lacZ* translational fusion was integrated at the *amyE* locus of *B. subtilis*. In these strains, chromosomal deletions inactivating *sacT* or *sacY* or the different genes of the PTS were created. The various strains were grown in minimal medium in the presence or absence of sucrose as the inducer (Table 4). Strains QB6023 and QB6024 are Δ(*sacX sacY*) and Δ*sacT* derivatives, respectively, of QB6010. QB6023 and QB6010 displayed inducible *lacZ* expression. The induction of *lacZ* expression was not observed in the Δ*sacT* strain QB6024. Thus, SacX and SacY have no effect on induction in the wild-type strain. However, in a *pts* mutant strain, SacY is fully active and allows the expression of *sacPA* through

TABLE 5. Effects of *pts* mutations on *sacP-lacZ* expression[a]

Strain	Relevant genotype	β-Galactosidase activity (Miller units/mg of protein)
QB6042	*sacT30 sacP-lacZ*	4,940
QB6052	*sacT30 sacP-lacZ* Δ(*sacX sacY*)	3,190
QB6064	*sacT30 sacP-lacZ* Δ(*sacX sacY*) Δ*sacP*	3,910
QB6065	*sacT30 sacP-lacZ* Δ(*sacX sacY*) Δ*ptsG*	4,265
QB6053	*sacT30 sacP-lacZ* Δ(*sacX sacY*) *ptsI7*	35
QB6050	*sacT30 sacP-lacZ* Δ(*sacX sacY*) Δ*ptsH*	40

[a] Cells were grown on CSK medium.

cross talk (Table 4). It has been previously shown that induction of *sacB* in the wild-type strain requires a high concentration of sucrose (22). Under these conditions, the expression of *sacPA* is abolished, probably because of catabolite repression. Furthermore, it was confirmed that SacT is a positive regulatory protein which controls *sacPA* operon expression. A deletion encompassing *ptsH*, *ptsI*, and *ptsG* was then introduced into these strains. *sacPA* was expressed constitutively in strains QB6014 and QB6027, probably because of the *sacY* gene product. Indeed, this constitutive expression in strain QB6026 was not observed. It is probable that when the PTS is inactivated, the SacY antiterminator is not phosphorylated and, therefore, is fully activated and probably interacts with the terminatorlike structure located downstream from the *sacPA* promoter, allowing transcription of the operon. In strain QB6026 containing deletions inactivating both the PTS and the SacY antiterminator, the *sacPA* operon was not expressed, indicating that SacT needs a component of the PTS to be an active antiterminator. To determine whether the *sacT30* gene product also requires the PTS for activity, *sacY* and *pts* null mutations were introduced into the *sacT30* strain. β-Galactosidase in cultures of these strains grown in minimal medium was assayed (Table 4). In strain QB6011 (*sacT30*), the uninduced level of expression of the *sacPA* operon was 10-fold higher than that observed in the induced QB6010 (*sacT*$^+$) strain. The high constitutive value was unaffected in strain QB6040 Δ(*sacX sacY*) *sacT30*, confirming that the *sacT30* gene product is responsible for this high level of expression. In strain QB6039 (*sacT30* Δ*pts*), an intermediate constitutive level of *sacPA* expression was observed and is probably due to SacY since it was completely abolished in a Δ*pts* Δ(*sacX sacY*) *sacT30* background (strain QB6041). These results show that in the absence of a functional PTS, the activities of both the *sacT30* and wild-type *sacT* gene products are abolished.

Roles of the genes of the PTS. The general proteins of the PTS, enzyme I and HPr, are required for the transport and phosphorylation of all PTS-sugars. Sugar specificity is determined by specific-membrane-bound enzymes II (SacP in the case of sucrose transport). In some cases, an enzyme III is involved (27). An enzyme III^{Glc} (*crr*) is involved in the uptake of sucrose in both *E. coli* and *Klebsiella pneumoniae* (20, 33). Enzymes III^{Glc} from gram-negative bacteria correspond to the C-terminal domain of an enzyme II^{Glc} in *B. subtilis* (14, 38). Moreover, this C-terminal enzyme III^{Glc}-like domain of enzyme II^{Glc} was shown to be involved in sucrose uptake via the sucrose-specific enzyme II, SacP (14, 38).

Mutations inactivating *ptsH* (HPr), *ptsI* (enzyme I), *ptsG* (enzyme II^{Glc}), or *sacP* (enzyme II^{Scr}) were introduced in a *sacT30* constitutive strain (Fig. 4). A deletion inactivating *sacX* and *sacY* was also introduced into each of these strains to avoid constitutive expression of the *sacPA* due to SacY (Table 5). In strain QB6064, from which *sacP* has been deleted, the constitutive expression of the *sacPA* operon is the same as that in the parental strain QB6052. In strain QB6065 containing a deletion of the C-terminal domain of enzyme II^{Glc}, the expression of the *sacPA* operon was also not significantly different from that of strain QB6052. These results clearly indicate that both enzyme II^{Scr} (*sacP*) and the enzyme III-like domain of *ptsG* are not involved in the expression of the *sacPA* operon. The introduction of either a point mutation (*ptsI7*) into enzyme I (QB6053) or a nonpolar internal deletion of the *ptsH* gene (strain QB6050) led to dramatic decreases in the expression of the operon, showing that enzyme I and HPr are necessary.

In strain QB6054 (Table 6), which contains an insertion modifying the transcriptional terminator, the operon is constitutively expressed. It was previously shown that this mutation allows the expression of the operon in absence of SacT in strain QB6056 (Table 2). As expected, the expression of the operon was unaffected in strain QB6060 from which *sacX* and *sacY* have also been deleted. A similar result was obtained with strain QB6063 deleted for *ptsG*, *ptsH*, and *ptsI*, confirming that the PTS is not required when the terminator is modified, for example, by the *sacPt2* mutation.

TABLE 6. SacT-independent expression of the *sacPA* operon in the *sacPt2* mutant[a]

Strain	Relevant genotype	β-Galactosidase activity (Miller units/mg of protein)
QB6054	*sacP-lacZ sacPt2*	350
QB6060	*sacP-lacZ* Δ(*sacX sacY*) *sacPt2*	355
QB6063	*sacP-lacZ* Δ(*sacX sacY*) Δ*pts*(*GHI*) *sacPt2*	375

[a] Cells were grown on CSK medium.

DISCUSSION

In *B. subtilis*, the expression of the *sacPA* and *sacB* genes is probably regulated by antitermination (4, 5). SacT, which controls the *sacPA* operon, shares extensive similarity with SacY, which regulates *sacB* gene expression. Both SacT and SacY are homologous to BglG, the antiterminator of the *bgl* operon of *E. coli* (5, 30, 35). Moreover, their putative targets are palindromic structures which are extremely similar (5). These properties could explain the observed cross talk between the two regulatory systems in *B. subtilis* (22, 36).

In this work, we used gene fusions to show that expression of the *sacT* gene is not autoregulated and is not controlled by the PTS or subject to a glucose effect. Since the expression of the *sacPA* operon was strongly reduced in the presence of glucose, a target of the glucose effect must be present in the 300-bp *Xmn*I-*Ssp*I restriction fragment which contains the promoter of the *sacPA* operon. This restriction fragment also contains a palindromic sequence downstream from the promoter. A mutational insertion between the two inverted repeats led to constitutive expression of the *sacPA* operon. This result strongly suggests that the inverted repeat acts as a transcriptional terminator, although no experimental data are yet available. Using this constitutive mutant in which the expression of the operon is independent of SacT, we showed

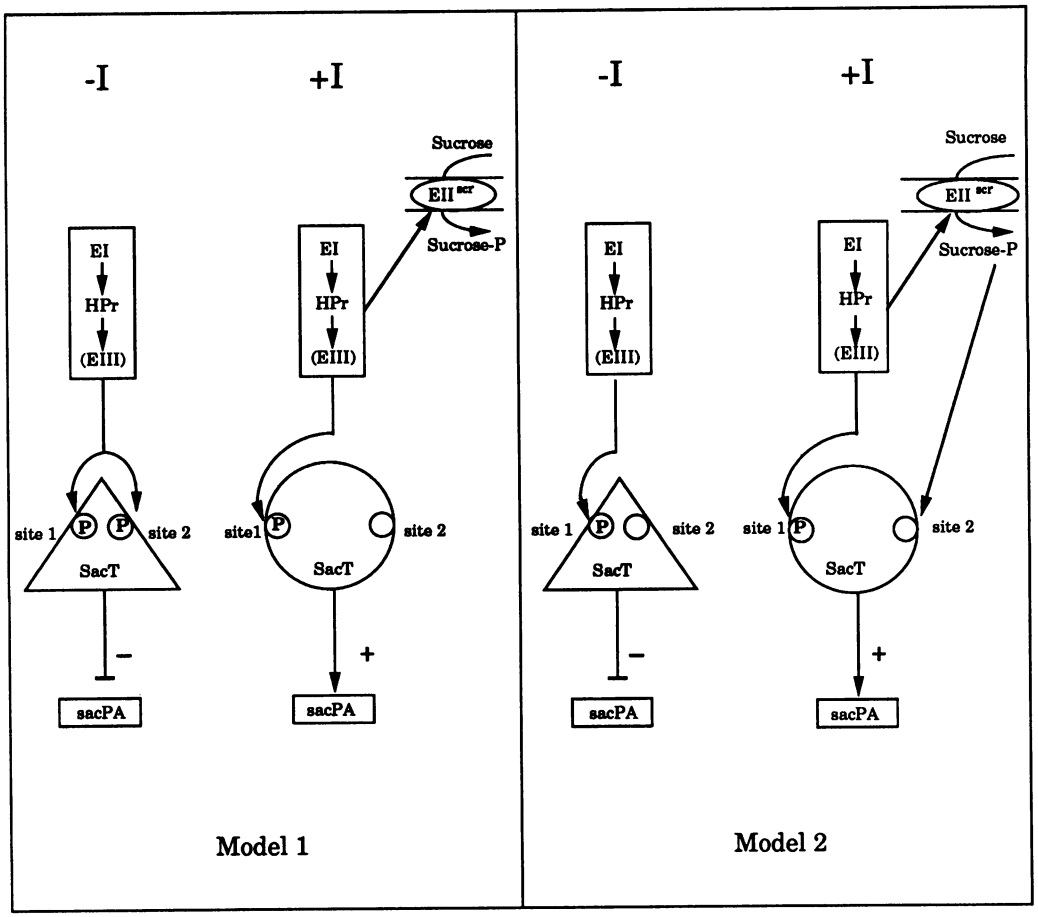

FIG. 5. Models proposed for the regulation of *sacPA* expression. In model 1, two sites of phosphorylation are present on SacT: site 1 is the activation site which is phosphorylated by components of the PTS (enzyme I, HPr, and possibly enzyme III^{Scr}); site 2 is the inactivation site which is phosphorylated in absence of inducer (−I). In the presence of sucrose (+I), site 2 is dephosphorylated as a consequence of sucrose transport and SacT becomes active. The role of site 2 is functionally equivalent to that proposed for the BglG antiterminator (1, 29). In model 2, in the absence of inducer (−I), the activation site 1 is phosphorylated while site 2 is not occupied; in the presence of sucrose (+I), sucrose-phosphate, formed during the transport of sucrose, acts as an internal inducer, activating SacT by interacting at site 2.

that the expression of the operon remained subject to a glucose effect.

It was previously shown that the PTS negatively regulates both *sacPA* and *sacB* gene expression (4, 5). A model was proposed for the control of SacY antiterminator activity. In this model, SacX is a minor but functional enzyme II^{Scr} which would phosphorylate SacY, thereby inactivating the antiterminator (4). In order to study a specific effect of SacT on the expression of the *sacPA* operon in *pts* mutants, it was essential to construct a series of strains deleted for *sacY*. Indeed, in a strain containing a deletion of *ptsH*, *ptsI*, and *ptsG*, the expression of the *sacPA* operon is constitutive. This study shows that SacY is responsible for the expression of *sacPA* in these *pts* mutants. Under these conditions, SacY could interact with the *sacPA* terminator. We also show that in *ptsI* or *ptsH* strains, both the wild-type SacT and the modified SacT30 antiterminators are not functional. This unexpected result strongly suggests that functional HPr protein and enzyme I are required for the activity of SacT. During the phosphorylation cascade, HPr is phosphorylated by enzyme I. Thus, the HPr protein is a candidate for interactions with the SacT antiterminator. It is therefore likely that HPr or a phosphorylated protein thereof phosphorylates the antiterminator SacT as a prerequisite for its activity. No specific enzyme III^{Scr} has been identified so far. However, an enzyme II^{Glc} (PtsG) with an enzyme III^{Glc}-like domain was shown to be involved in sucrose uptake via the enzyme II^{Scr} (SacP) (38). In this study, we showed that both the *ptsG* and *sacP* gene products are not required for SacT activity. Still, we cannot exclude that a specific enzyme III^{Scr} is involved as an intermediate in the phosphorylation cascade. In this case, this putative enzyme III^{Scr} would activate SacT by protein-protein contact or by phosphorylation on a specific site (Fig. 5). The role of sucrose as an inducer remains ambiguous. We can postulate that in the absence of sucrose, SacT is presumably phosphorylated at a second site, which leads to its inactivation as proposed for BglG (1, 29) and SacY (4). This step would require an unidentified enzyme II^{Scr} or the above postulated enzyme III^{Scr}. In the presence of sucrose, this second site of SacT would be dephosphorylated and SacT thereby would be fully activated, while sucrose would be phosphorylated during its uptake (Fig. 5, model 1). An alternative to this phosphorylation hypothesis is the direct interaction of SacT, phosphorylated by enzyme I and HPr, with an internal inducer (Fig. 5, model 2).

In conclusion, the PTS exerts different effects on *sacB* and *sacPA*. The general proteins of the PTS are not required for the activity of SacY, while they are necessary for SacT activity.

Confirmation of the phosphorylation of SacT by the PTS components will be necessary, by using purified proteins.

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