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Induction of Saccharolytic Enzymes by Sucrose in *Bacillus subtilis*: Evidence for Two Partially Interchangeable Regulatory Pathways

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Sucrose induces two saccharolytic enzymes in *Bacillus subtilis*, an intracellular sucrase and an extracellular levansucrase, encoded by *sacA* and *sacB*, respectively. It was previously shown that the *sacY* gene encodes a positive regulator involved in a sucrose-dependent antitermination upstream from the *sacB* coding sequence. We show here that the *sacY* product is not absolutely required for *sacB* induction: a weak but significant induction can be observed in strains harboring a *sacY* deletion. The *sacY*-independent induction was altered by mutations located in the *sacP* and *sacT* loci but was observed in both *sacU*$^+$ and *sacU32* genetic backgrounds. These results suggest that *B. subtilis* has two alternative systems allowing *sacB* induction by sucrose. Both systems also seem to be involved in *sacA* induction.

Sucrose induces the synthesis of at least three proteins in *Bacillus subtilis* (for a review, see reference 6): an intracellular sucrase (gene *sacA*), an extracellular levansucrase (LS) (gene *sacB*), and a sucrose-specific permease (enzyme II^{Suc}, gene *sacP*) belonging to the phosphorylating transport system (for a review, see reference 10). *sacP* and *sacA* appear to be organized in an operon (3) and are not linked to *sacB*. The *sacT* locus was identified by a single *sacT30* mutation linked to the putative *sacP-A* operon which renders both *sacA* and *sacP* constitutive (6).

sacB regulation is beginning to be understood. The coding sequence is preceded by a 400-base-pair regulatory region called *sacR* which contains the promoter and the targets for several regulators. The products of the *sacU* and *sacQ* genes activate transcription initiation from the promoter. Their target is upstream of the promoter, but a direct interaction has not been shown (1, 4, 16). There is a transcriptional terminator involved in *sacB* induction by sucrose just downstream of the promoter. Mutations or deletions altering this structure make *sacB* constitutive (13). Furthermore, it has been shown that the *sacB* promoter is constitutive but that transcripts stop at the terminator in the absence of sucrose and extend past the terminator only in the presence of sucrose (12). Therefore, it was proposed that *sacB* induction involved an original type of transcriptional attenuation, and it was suggested that a sucrose-dependent regulator modulated termination at the *sacR* level (12, 13).

The *sacS* locus, linked neither to *sacA* nor to *sacB*, was identified by several kinds of mutations, some of which render both these genes constitutive (6). This locus was recently cloned and shown to encode a positive regulator of *sacB* expression. It was observed that this regulator was no longer required for *sacB* expression when the *sacR* terminator was inactivated by mutation. Therefore, it was suggested that *sacS* encodes an antiterminator interacting with the *sacR* transcript (2). The *sacS* locus was sequenced and shown to contain two open reading frames, *sacX* and *sacY* (460 and 280 codons, respectively), that might constitute an operon (17; M. Zukowski et al., manuscript in preparation) (see Fig. 2B). Preliminary evidence suggests that the *sacX* product negatively controls the product of *sacY* (2; M. Steinmetz, unpublished results). *sacY*, downstream from *sacX*, encodes the putative antiterminator which is highly homologous with an *Escherichia coli* putative antiterminator, the product of the *bglC* gene (11) (Fig. 1). Convincing evidence that BglC was involved in the induction of the *bgl* operon by interacting with two terminators that show sequence homology with the *sacR* terminator was obtained (7, 8, 11). A direct interaction of SacY with the transcription apparatus has not been demonstrated, but the existence of an intermediary regulator between SacY and *sacR* seems very unlikely: it was shown that a positive regulator was limiting for induction of a *sacR*::*xylE* fusion present on a multicopy plasmid and that cloning *sacY* on the plasmid relieved this limitation (17; Zukowski et al., in preparation).

It had been previously suggested that the *sacS* locus was involved in induction of both *sacA* and *sacB*. This hypothesis was supported by mutations within *sacS* which make both genes constitutive. However, other observations were more difficult to reconcile with such a simple model: first, sucrose analogs such as glucosido-sorboside were good inducers of *sacA* but poor inducers of *sacB*; second, *sacA* and *sacB* were fully induced with different minimal sucrose concentrations; and third, a second class of *sacS* mutations was shown to abolish the expression of *sacB* without significantly affecting expression of *sacA* (6). These mutations were recently mapped within *sacY* (Steinmetz, unpublished results). In this paper, we present data allowing a clarification of this apparent paradox: we show that the genes belonging to the *sacS* locus are not absolutely required for *sacB* induction. Our observations strongly suggest that *B. subtilis* has two regulatory systems which allow induction of saccharolytic enzymes.

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains used are described in Table 1. QB4503 was kindly provided by G. Rapoport; a chloramphenicol resistance gene was substituted for an internal part of the *sacP* gene (about 30% of the coding sequence) within its chromosome (see Fig. 4). GM122 contains the *lacZ* gene under the control of the *sacR* regulatory region and substituted for part of the *sacB* gene (Fig. 2A). This *sacR*::*lacZ* fusion is identical to that present on plasmid pLG131 (1). GM152 is a GM122 derivative which carries a

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BglC  MNMQITKILNNNVVVVIDDQQREKVVMGRGIGFQKRAGERINSSGIEKEY
SacY    MKIKRILNHNAIVVK-DQNEEKILLGAGIAFNKKKNDIVDPSKIEKTF
        = = -=== = -==  ==- ==---= == =--=  - -  = === -

        ALSSHELNGRLSELLSHIPLEVMATCDRIISLAQERLG-KLQDSIYISLT
        IRKDTPDYKQFEEILETLPEDHIQISEQIISHAEKELNIKINERIHVAFS
                    =-=  -= - -   - === =   =  =--- = -  -

        DHCQFAIKRFQQNVLLPNPLLWDIQRLYPKEFQLGEEALTIIDKRLGVQL
        DHLSFAIERLSNGMVIKNPLLNEIKVLYPKEFQIGLWARALIKDKLGIHI
        ==  === =  - --- ==== -=  ========-=  =  -=  -==- -

        PKDEVGFIAMHLVSA-QMSGNMEDVAGVTQLMREMLQLIKFQFSLNYQEE
        PDDEIGNIAMHIHTARNNAGDMTQTLDITTMIRDIIEIIEIQLSINIVED
        = ==-= =====- -= -  = =     -= --=--- -=  = =-=  =-

        SLSYQRLVTHLKFLSWRILEHASINDSDESLQQAVKQNYPQAWQCAERIA
        TISYERLVTHLRFAIQHIKAGESIYELDAEMIDIIKEKFKDAFLCALSIG
        ---= =======-=    =    == - = -  -=  -  =  ==  =

        IFIGLQYQRKISPAEIMFLAINIERVRKEH
        TFVKKEYGFEFPEKELCYIAMHIQRFYQRSVAR
         =-  =       =- --=- = =
```

FIG. 1. Homology between the *bglC* and *sacY* products (278 and 280 residues, respectively). - and =, Identical (34%) and similar (20%) amino acids, respectively.

2.1-kilobase deletion (*sacSΔ3*) removing the 3′ end of *sacX* and all of *sacY* (Fig. 2B).

DNA manipulation, plasmids, and transformation. The standard procedures used in this study and plasmids pLS50, pLG131, and pSL42 were described previously (1, 2, 15). The construction of pLG231 and pSL75-C is described in the legend to Fig. 2.

Media and phenotypical characterization. CG medium is the C mineral medium previously described (1) supplemented with 10 mg of glucose per ml. CgCH medium is C medium supplemented with glucitol and casein hydrolysate (10 and 2 mg/ml, respectively). Cultures were inoculated at 5 Klett units (600 nm) with precultures exponentially growing in the same medium. The precultures were inoculated with cells from colonies grown overnight on LB plates (9). Precultures in CG medium were inoculated at low cell density and grown overnight. Precultures in CgCH medium were inoculated at 5 to 10 Klett units and grown for 3 h. Precultures and cultures were grown at 37°C with vigorous shaking.

sacA and *lacZ* expression was induced by sucrose addition (75 mM final concentration, except when stated otherwise) to cultures at 15 Klett units. Samples were taken at 150 Klett units, cells were lysed, and enzymatic activities were assayed as previously described (1, 5).

TABLE 1. *B. subtilis* strains used

Strain	Genotype[a]	Origin or reference[b]
168	*trpC2*	Our collection
QB112	*sacA331 sacU32*	Our collection
QB4503	*trpC2 sacP::cat*	G. Rapoport[c]
GM122	*trpC2 sacR::lacZ*	This work (Fig. 2)
GM130	As GM122 with *sacU32* and *trp*$^+$	QB112 tf into GM122
GM152	As GM122 with *sacSΔ3*	This work (Fig. 2)
GM153	*leu-8 sacT30*	Our collection
GM170	As GM122 with *sacT30* and *trp*$^+$	GM153 tf into GM122
GM177	As GM130 with *sacSΔ3*	pSL75-C tf into GM130
GM190	As GM170 with *sacSΔ3*	pSL75-C tf into GM170
GM250	As GM122 with *sacP::cat*	QB4503 tf into GM122
GM253	As GM152 with *sacP::cat*	QB4503 tf into GM152

[a] The structures of the *sacP::cat*, *sacSΔ3*, and *sacR::lacZ* alleles are shown in Fig. 2 and 4.
[b] tf, Transformation.
[c] Unité de Biochimie Microbienne, Institut Pasteur, Paris, France.

RESULTS

Inducible expression of a *sacR::lacZ* fusion in a strain deleted for *sacY*. We constructed strains containing *sacR::lacZ* fusions which were under the control of the regulatory region *sacR* (Fig. 2A). Thus, the regulation of *sacA* and *sacB* could be easily monitored in these strains by distinct activities, sucrase and β-galactosidase, respectively. Derivatives of these strains which contained a deletion, *sacSΔ3*, were constructed, removing all of *sacY* and the 3′ end of *sacX* (Fig. 2B).

Sucrose induced both *sacA* and *lacZ* (*sacB*) in the *sacS*$^+$ strain GM122 (Table 2). Expression of *lacZ* in strain GM152 (*sacSΔ3*) was influenced by the growth medium. No expression of *lacZ* was observed in mineral medium containing glucose as the carbon source (CG medium), whereas a reduced but significant induction was reproducibly observed when GM152 was grown in the presence of glucitol and casein hydrolysate (CgCH medium). *sacA* was inducible in both strains and under both growth conditions; however, a lower sucrase level was observed in the *sacSΔ3* mutant when the mutant was grown in CG medium. A nearly normal *sacA* expression was observed when the *sacSΔ3* mutant was grown in CgCH medium (Table 2). Thus, a *sacY*-independent mechanism allowed both *sacA* and *lacZ* (*sacB*) induction in the *sacSΔ3* mutant under some growth conditions, but this mechanism was inefficient for *lacZ*. These results suggest the following hypothesis. *B. subtilis* would have two systems that allow induction of saccharolytic enzymes: a *sacY*-dependent one, efficient for *sacB* induction; and a *sacY*-independent one, with low efficiency for *sacB* but greater efficiency for *sacA*. The second system would be more functional under some growth conditions (CgCH medium) than under others (CG medium).

It was previously reported that the parameters of *sacB* and *sacA* induction were different (6). This was confirmed for strain GM122, in which *lacZ* (*sacB*) and *sacA* reached their maximal expressions with 30 mM and less than 1 mM sucrose, respectively (Fig. 3A). Interestingly, the maximal expression of both genes was reached with less than 1 mM sucrose in GM152 (*sacSΔ3*).

Reexamination of the phenotype of the *sacT30* mutant. The *sacT30* mutation, linked to *sacP* and *sacA*, makes expression of these genes constitutive. Therefore, it was suggested that *sacT* was the *cis*-regulatory region of the putative *sacA-P* operon (6). However, it was subsequently observed that *sacB* was semiconstitutive but still overinducible by sucrose in a *sacT30 sacU32* double mutant (M. Pascal, thèse de Doctorat d'Etat, Université Paris 7, 1976). We confirmed this observation with a derivative of GM122 (*sacU*$^+$) containing the *sacT30* mutation. In this strain, GM170, *lacZ* (*sacB*) and *sacA* were constitutive and *lacZ* was overinducible by sucrose. The overinduction was rather weak but reproducibly observed when the strain was grown in CgCH medium. A very clear overinduction was observed in CG medium. Both genes were still constitutive in GM190, a *sacT30 sacSΔ3* double mutant, but no overinduction of *lacZ* was observed (Table 3). Thus the constitutive *lacZ* expression in the *sacT30* mutant is *sacY* independent. Furthermore, it was observed that, as in the *sacT*$^+$ background, the *sacY*-independent expression of *lacZ* in a *sacT30* mutant was higher in CgCH medium than in CG medium (Table 3).

Role of *sacP* in *sacB* induction. It was reported previously that *sacP* mutants were deficient in sucrose transport and in expression of both *sacA* and *sacB* (5). To confirm this observation, we introduced a *sacP::cat* insertion (Fig. 4) into

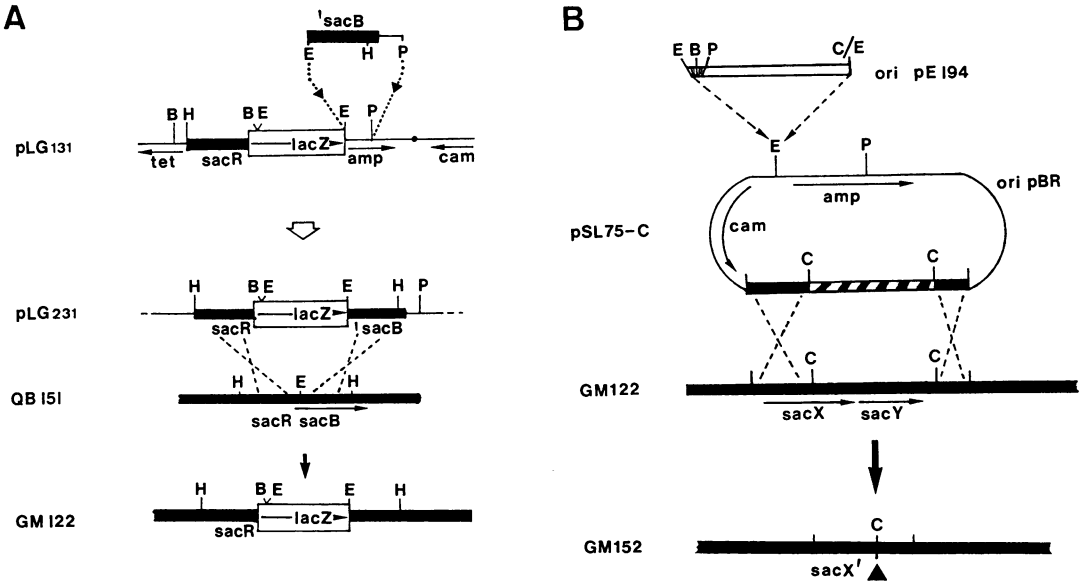

FIG. 2. Construction of strains GM122(*sacR*::*lacZ*) and GM152(*sacSΔ3*). (A) An *Eco*RI-*Pst*I pLS50 fragment which contains the *sacB* 3′ end was inserted downstream of *lacZ* into pLG131, giving pLG231. A mixture of DNA from pLG231 and strain 168 was used to transform strain QB151 (*metC3*). The transformant GM122, resulting from the double crossover shown, was selected as Met^+ $SacB^-$ $LacZ^+$ on mineral medium containing sucrose and X-Gal. (B) pSL75-C was derived from pSL42 in two steps. First, an *Eco*RI cartridge containing the pE194 thermosensitive origin of replication was inserted into pSL42, giving pSL85 (the cartridge is the *Cla*I-*Pst*I 1.7-kilobase fragment of pE194 ligated with pUC8 polylinker at the level of the *Pst*I site and with an *Eco*RI linker at the level of the *Cla*I site). Second, the 2.1-kilobase *Cla*I fragment (*sacX* 3′ end and *sacY* [▨]) was deleted. pSL75-C was used to transform GM122. Chloramphenicol-resistant transformants were selected at 45°C (nonpermissive temperature for pE194 replication). This resulted in integration of pSL75-C into the chromosome. About 5% of the transformants did not express *lacZ* on sucrose medium because of introduction of the *Cla*I deletion (*sacSΔ3*) into the chromosome through conversion. One of these transformants was grown at the permissive temperature (this results in plasmid excision). A second growth cycle at 45°C, in the absence of chloramphenicol, resulted in the plasmid-free strain GM152. C, E, H, and P, *Cla*I, *Eco*RI, *Hin*dIII, and *Pst*I restriction sites, respectively.

strain GM122 by transformation with DNA extracted from strain QB4503. It was observed that *lacZ* was still inducible in the resulting strain GM250 (*sacP*::*cat*), at a level corresponding to about 10% of that observed in the $sacP^+$ parent GM122 (Table 3). *sacA* expression was abolished in GM250, but no interesting conclusion could be deduced from this observation, because the *cat* gene inserted into *sacP* must have a polar effect on *sacA* expression in this strain (Fig. 4). In the *sacSΔ3 sacP*::*cat* double mutant, GM253, *lacZ* induction was completely abolished (Table 3).

***sacY*-independent induction in a *sacU32* genetic background.** The *sacU32* mutation strongly increases *sacB*-inducible expression (1, 6, 13). The *sacU* gene is likely to encode an activator that directly or indirectly stimulates transcription initiation from the *sacB* promoter (4, 16). Expression of *lacZ* was studied in GM122 derivatives carrying the *sacU32* mutation. This mutation resulted in a 150-fold increase of *lacZ* expression (compare Fig. 3A and B). In the *sacU32* context, the *sacSΔ3* mutation strongly decreased *lacZ* expression (Fig. 3B). Furthermore, as with the $sacU^+$ background, maximal *lacZ* induction was reached with 30 mM and about 1 mM sucrose in the $sacY^+$ and *sacY* mutant strains, respectively (Fig. 3B).

TABLE 2. Sucrase and β-galactosidase expression in GM122 ($sacS^+$) and GM152 (*sacSΔ3*)[a]

Strain and status[b]	CG medium		CgCH medium	
	β-galactosidase	sucrase	β-galactosidase	sucrase
GM122				
−I	<0.1	<0.3	<0.1	<0.3
+I	5.2	2.6	6.2	4.9
GM152				
−I	<0.1	<0.3	<0.1	<0.3
+I	<0.1	0.7	1.0	3.9

[a] Growth and induction by sucrose addition were performed as described in Materials and Methods.
[b] −I, Uninduced; +I, induced.
[c] β-Galactosidase activities are expressed in Miller units (9). Sucrase activities are expressed in sucrase units per liter at 100 Klett units (1 sucrase unit is 1 μmol of glucose liberated per minute).

DISCUSSION

It was previously suggested that the *sacS* locus played a central role in induction by sucrose of both *sacA* and *sacB*. This hypothesis was supported by the characterization of mutations within this locus which rendered both genes constitutive. Other observations, however, seemed to contradict this view: higher inducer concentration was required to obtain full induction of *sacB*, and sucrose analogs were good inducers of *sacA* but not of *sacB* (6). It must be noted that the K_m of LS for sucrose is also high (35 mM [6]); therefore, it could be proposed that LS is involved in its own induction through synthesis of a secondary inducer. This synthesis would be dependent on sucrose concentration and could not occur with sucrose analogs. But this hypothesis must be rejected, since expression of the *sacR*::*lacZ* fusion, which measures LS regulation, was also dependent on sucrose concentration in strains such as GM122 and GM130, which contain a deletion of the *sacB* gene (Fig. 3).

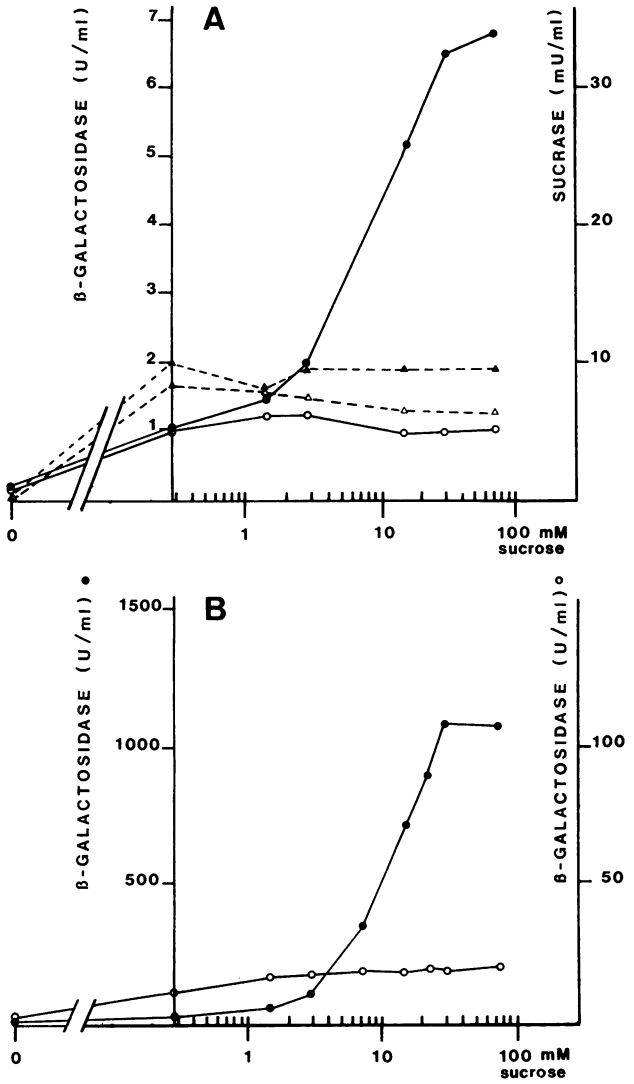

FIG. 3. Sucrase and β-galactosidase induction in GM122 derivatives according to inducer concentration. Strains were grown in CgCH medium, and various amounts of sucrose were added to fractions of the culture. Extracts were prepared and enzymatic activities were assayed as described in Materials and Methods. Circles and triangles, β-Galactosidase and sucrase activities, respectively; closed and open symbols, *sacS*$^+$ and *sacSΔ3* strains, respectively. (A) *sacA* and *lacZ* expression in GM122(*sacU*$^+$) and its *sacSΔ3* derivative, GM152. (B) *lacZ* induction in GM130(*sacU32*) and GM177(*sacU32 sacSΔ3*). Note that GM130 (●) and GM177 (○) activities are not on the same scale.

We show here that the *sacSΔ3* deletion that removes all of the *sacY* open reading frame does not abolish induction of either the *sacA* gene or the *sacR*::*lacZ* fusion (Table 2). The *sacY*-independent induction of the fusion was low but observed in both *sacU*$^+$ and *sacU32* backgrounds; the effect of the deletion was significant only at high sucrose concentrations (Fig. 3). In this way, SacY is not absolutely required for induction of saccharolytic enzymes. This could signify that SacY would be an accessory regulator indirectly controlling this induction by interacting with the regulator which directly modulated termination at the *sacR* level. Several lines of indirect evidence make this hypothesis unlikely (see the introduction). An alternative interpretation could be proposed: in addition to the system involving SacY, *B. subtilis* possesses a second system which allows induction of *sacA* and *sacB*. The *sacT30* mutation seems to affect this second system: *sacT30* rendered *lacZ* (*sacB*) semiconstitutive, and this phenotype was *sacY* independent, since it was observed in a *sacSΔ3* background (Table 3). It is unclear how the *sacT* locus is involved in this mechanism, since this locus has not yet been cloned. But it is clear that *sacT* controls *sacB* through a diffusible regulator. Therefore, it is unlikely that *sacT* is an operatorlike site controlling the *sacP-A* operon, as suggested previously (6), unless this operon contains a gene coding for a diffusible regulator. It was not surprising that *sacP* was involved in induction of saccharolytic enzymes, since this gene encodes an enzyme II^{Suc} that catalyzes sucrose permeation (3). Since *lacZ* residual expression observed in the *sacSΔ3* background was abolished by the *sacP*::*cat* insertion (Table 3), it could be concluded that *sacP* was involved in the *sacY*-independent system.

TABLE 3. *sacA* and *lacZ* expression in GM122 derivatives[a]

Strain	Relevant genotype	β-Galactosidase activity		Sucrase activity	
		−I	+I	−I	+I
GM122	Wild type	<0.1	6.2	<0.3	4.9
GM152	*sacSΔ3*	<0.1	1.0	<0.3	3.9
GM170	*sacT30*	2.1 (0.8)	4.0 (9.4)	7.3	5.2
GM190	*sacT30 sacSΔ3*	1.5 (0.6)	1.5 (0.7)	8.0	4.5
GM250	*sacP*::*cat*	<0.1	0.4	<0.3	<0.3
GM253	*sacP*::*cat sacSΔ3*	<0.1	<0.1	<0.3	<0.3

[a] Experimental procedures were similar to those described in Table 2. −I, Uninduced; +I, induced. Values are for cultures in CgCH medium; values in parentheses are for cultures in CG medium. Activities are expressed as in Table 2.

sacA was weakly affected by the *sacSΔ3* deletion. On the other hand, this deletion strongly decreased the expression of the *sacR*::*lacZ* fusion (Table 3). These observations suggest that SacY is more specifically involved in *sacB* induction and, at least under some growth conditions, dispensable for *sacA* induction. The second system would be more specifically involved in *sacA* induction but could partially replace *sacY* when this gene is deleted. As expected, when *sacY* was deleted, the residual induction of *lacZ* was maximal at low sucrose concentrations, like *sacA* induction in both *sacY*$^+$ and *sacY* mutant contexts (Fig. 3).

It is easy to realize that this double system might be worthwhile for *B. subtilis* because sucrase and LS are involved in quite different pathways of sucrose metabolism. Sucrase is responsible for intracellular catabolism of sucrose and is induced at low substrate concentrations (5). These features are those of classical catabolic enzymes. LS allows extracellular sucrose metabolism and is fully induced only at

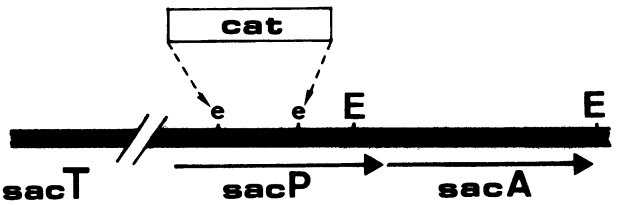

FIG. 4. The *sacP*::*cat* insertion corresponds to the substitution of an internal part of *sacP* for a chloramphenicol gene (*cat*). The *sacT* locus is linked to *sacP*, but its precise position is unknown. e, *Eco*RV restriction sites used to insert *cat*; E, *Eco*RI sites.

high sucrose concentrations, similar to its K_m for sucrose (6; this paper). On the other hand, it is more difficult to understand why *B. subtilis* has two metabolic pathways for sucrose and to understand the role of levan, the specific product of LS. In the fully induced wild-type strain, the LS contribution to sucrose assimilation is only 5% of the sucrase contribution, at least under laboratory conditions (6). This could signify that LS is not a catabolic enzyme or that sucrose is not the only signal required to induce LS to a significant level. The *sacU32* mutation results in a great increase in LS expression (6); therefore, it could be suggested that one of the roles of *sacU* is to recognize specific ecological niches where levan synthesis could be useful to *B. subtilis* cells.

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