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Multiple Phosphorylation of SacY, a *Bacillus subtilis* Transcriptional Antiterminator Negatively Controlled by the Phosphotransferase System*

Pablo Tortosa‡, Stéphane Aymerich‡, Cordula Lindner§¶, Milton H. Saier, Jr.‖, Jonathan Reizer‖, and Dominique Le Coq‡*

From the ‡Laboratoire de Génétique des Microorganismes, Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique ERS 567, F-78850 Thiverval-Grignon, France, the §Institut de Biologie et Chimie des Protéines, Centre National de la Recherche Scientifique UPR 412, F-69367 Lyon Cedex 07, France, and the ‖Department of Biology, University of California at San Diego, La Jolla, California 92093–0116

The *Bacillus subtilis* SacY transcriptional antiterminator is a regulator involved in sucrose-promoted induction of the *sacB* gene. SacY activity is negatively controlled by enzyme I and HPr, the general energy coupling proteins of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), and by SacX, a membranal protein homologous to SacP, the *B. subtilis* sucrose-specific PTS-permease. Previous studies suggested that the negative control exerted by the PTS on bacterial antiterminators of the SacY family involves phosphoenolpyruvate-dependent phosphorylation by the sugar-specific PTS-permeases. However, data reported herein show direct phosphorylation of SacY by HPr(His~P) with no requirement for SacX. Experiments were carried out to determine the phosphorylatable residues in SacY. *In silico* analyses of SacY and its homologues revealed the modular structure of these proteins as well as four conserved histidines within two homologous domains (here designated P1 and P2), present in 14 distinct mRNA- and DNA-binding bacterial transcriptional regulators. Single or multiple substitutions of these histidyl residues were introduced in SacY by site-directed mutagenesis, and their effects on phosphorylation and antitermination activity were examined. *In vitro* phosphorylation experiments showed that SacY was phosphorylated on three of the conserved histidines. Nevertheless, *in vivo* studies using cells bearing a *sacB'-lacZ* reporter fusion, as well as SacY mutants lacking the phosphorylatable histidyls, revealed that only His-99 is directly involved in regulation of SacY antitermination activity.

The *Bacillus subtilis* SacY protein is a transcriptional antiterminator involved in sucrose-promoted induction of the *sacB* gene, encoding levansucrase (1–4). Transcription initiates constitutively from the *sacB* promoter, but in the absence of sucrose, most transcripts terminate at a rho-independent transcriptional terminator located in the leader region preceeding the *sacB* structural gene. In the presence of sucrose, SacY is activated and stabilizes a stem-loop structure of a ribonucleic acid antiterminator, thereby preventing alternative formation of the overlapping transcriptional terminator (5). SacY activity is negatively controlled by enzyme I (EI)[1] and HPr, the general energy coupling proteins of the phosphoenolpyruvate:sugar phosphotransferase system (PTS), and by SacX, a protein homologous to sucrose-specific PTS permeases (6, 7).

The PTS catalyzes both the transport and the phosphorylation of numerous carbohydrates in bacteria. Energy for these coupled reactions is provided by phosphoenolpyruvate (PEP). The phosphoryl group of PEP is sequentially transferred to the incoming sugar *via* EI, HPr, and two domains in each of the sugar-specific permeases. The modular carbohydrate-specific permeases are generally comprised of three proteins or domains, EIIA, EIIB, and EIIC (8). The EIIC protein or domain is the integral membrane permease, whereas EIIA and EIIB are hydrophilic, peripherally membrane-associated proteins or domains that are phosphorylated during the phosphoryl transfer cascade. EIIB~P transfers its phosphate to the incoming sugar during its translocation across the membrane (for reviews, see Refs. 9 and 10). SacX is thought to be an EIIBC protein; the corresponding EIIA protein has not been characterized to date.

Our long term interest in the multifaceted controls exerted by the PTS led us to investigate the molecular mechanism involved in PTS-mediated regulation of transcriptional antitermination by SacY. Previous genetic data (7) and comparison with results reported for the homologous BglG antiterminator of *Escherichia coli* (11–13) suggested that this control involves a cascade of phosphorylations. The *E. coli bgl* operon, required for transport and hydrolysis of β-glucosides, is inducible by β-glucosides through a BglG-dependent antitermination mechanism (14, 15). Activity of BglG is negatively controlled by BglF, a β-glucoside permease with EIIBCA domain structure, that has been reported to phosphorylate BglG at an unknown residue in response to the external level of inducer (11–14). Furthermore, phosphorylation of BglG has been shown to prevent its dimerization. Consequently the PTS was proposed to control the antitermination activity of BglG by modulating the level of its active, unphosphorylated, dimeric form (16).

Two other antiterminators in *B. subtilis*, in addition to SacY, belong to the BglG family: SacT mediates induction of the

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¶ Present address: Institut für Mikrobiologie, Ernst-Moritz-Arndt Universität, D-17487 Greifswald, Germany.

** To whom correspondence should be addressed. Tel.: 33-01-30-81-54-45; Fax: 33-01-30-81-54-57; E-mail: lecoq@cardere.grignon.inra.fr.

[1] The abbreviations used are: EI, enzyme I; PTS, phosphoenolpyruvate:sugar phosphotransferase system; PEP, phosphoenolpyruvate; GST, glutathione *S*-transferase; IPTG, isopropyl-β-D-thiogalactopyranoside.

sacPA operon by sucrose (17), and LicT mediates induction of the *licTS* and *bglPH* operons by β-glucosides (18, 19). Interestingly, SacT and LicT are not active in mutants lacking EI (*ptsI*) or HPr (*ptsH*) (20–22). Thus, PTS-mediated phosphorylation appears to exert positive control over SacT and LicT antitermination activities. Positive control by the PTS has been also demonstrated for LevR, a *B. subtilis* DNA-binding transcriptional regulator involved in fructose-promoted induction of the levanase operon (23, 24). Recent experiments have shown that SacT and LevR are phosphorylated by PEP in a PTS-catalyzed reaction that depends only on EI and HPr. These observations suggested that phosphorylation is involved in PTS-mediated positive control of these transcriptional regulators (4, 24). We note however that despite the sequence conservation between the antiterminators of the BglG family, SacY is not subject to PTS-mediated positive control since *sacB* is constitutively expressed in a *ptsHI* mutant (7). This fact renders SacY a good model protein for analysis of PTS-mediated negative control of antitermination.

In this manuscript, we show that SacY is readily phosphorylated by PEP in a PTS-catalyzed reaction that depends only on EI and HPr. Additionally, computer-aided analyses led us to identify histidyl residues that are highly conserved in the modular SacY and in 13 distinct RNA- and DNA-binding proteins that bear duplicated SacY-like domains. Utilizing site-directed mutants of these conserved histidyl residues, we provide evidence demonstrating that His-99, His-207 and His-269 of SacY are phosphorylated by HPr(His~P). Finally, we present data suggesting that PTS-dependent inactivation of SacY activity is mainly due to phosphorylation of His-99. Altogether, the data presented define the role of PTS-catalyzed phosphorylation of SacY in the modulation of its transcriptional antitermination activity. Since SacY-like domains as well as phosphorylatable histidyl residues are highly conserved in 14 distinct mRNA-binding and DNA-binding proteins, the reported data provide guidelines for studies of PTS-dependent transcriptional regulation by a unique family of functionally distinct regulatory proteins.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—A fragment carrying the entire *sacY* sequence, flanked with *Bam*HI and *Eco*RI restriction sites, was generated by PCR with the DYB (5'-CGCGGATCCATGAAAATTAA-AAGAATCTTAAATC-3') and FYE (5'-CCGGAATTCAGCGTGCGACT-GACCGTTGG-3') primers on pSL90 (7). Following digestion with *Bam*HI and *Eco*RI, the 847-base pair fragment was cloned into pBluescript SK⁻ (Stratagene, La Jolla, CA) generating pIC437. The CAT (His) codon at position 99, 158, 207, or 269 was substituted with a TAT (Tyr) codon by site-directed mutagenesis using the Muta-gene M13 *in vitro* mutagenesis kit (Bio-Rad, Hercules, CA). *sacY* alleles carrying two or three of these His to Tyr mutations were constructed by successive site-directed mutageneses or, when possible, by exchange of a relevant restriction fragment. The wild-type and mutated *sacY* genes were then cloned into the *B. subtilis* pSL90 integrative plasmid (7), generating a collection of plasmids that were used to integrate the different alleles at the *sacY* locus. For this, the *Bst*BI/*Acc*I fragment of pSL90, carrying the 3' part of *sacY*, was replaced with the *Bst*BI/*Eco*RV fragment of pIC437 or one of its derivatives carrying a *sacY* mutant allele.

The *Bam*HI/*Eco*RI fragment from pIC437 or its derivatives was inserted into pGEX-2T (Pharmacia, Uppsala, Sweden) to give a plasmid overproducing the wild-type or a mutant glutathione *S*-transferase (GST)-SacY fusion protein. pGEX-2T derivatives overproducing the wild-type or the mutated P1 and P2 domains of SacY were constructed as follows. DNA fragments carrying the wild-type or the mutated sequences encoding the P1 and P2 domains flanked with *Bam*HI and *Eco*RI restriction sites, were generated by PCR using pIC437 or its derivatives with the primers DYP1B (5'-CGCGGATCCACATTGCCTG-AAGACCAC-3') and FYP1E (5'-CCGGAATTCAGGCATTGTTTCTT-GCTGTG-3') (for the P1 domain encoding sequence) or DYP2B (5'-CGCGGATCCGGCGATATGACACAAAC-3') and FYE (for the P2 domain encoding sequence). These fragments were then digested with

*Bam*HI and *Eco*RI, and cloned into pGEX-2T. The entire *sacY* gene as well as the P1 and P2 encoding sequences in the different plasmids were verified by sequencing after site-directed mutagenesis or PCR.

Plasmid pIC424, which carries the *B. subtilis pts* operon with a *ptsI::spe* disruption, was derived from pTS20 (25) by insertion of a spectinomycin resistance gene, *i.e.* a *Sma*I fragment from pIC156 (26), into the *Sst*I site of *ptsI*. pSL151 is a *B. subtilis* replicative plasmid, carrying the pUB110 replication origin, the chloramphenicol resistance *cat* gene, and the *sacX* gene under the control of the *spac* promoter (27).

E. coli strains used for plasmid construction, mutagenesis and protein overproduction were DH5α, CJ236, and BL21(DE3) respectively (28). *B. subtilis* GM152 (*trpC2 sacB'-lacZ sacXYΔ3*) was described previously (7). The *B. subtilis* strain GM1288 (*sacXYΔ3 sacBΔ23 sacTΔ4 amyE::bglP'-lacZ Δ(licS-bglP)::aphA3*) is deleted of all known antiterminator genes and derived from GM1271 (18). *B. subtilis* GM1320 and GM1386 were derived from GM1288 by an in-frame deletion of the *Dra*I/*Hpa*I fragment of *ptsH* (deletion of codons 6–34) and by introduction of the *ptsI::spe* disruption present in plasmid pIC424, respectively. The *B. subtilis* strains GM1288X, GM1320X, and GM1386X were obtained by transformation of GM1288, GM1320, and GM1386, respectively, with pSL151.

Media, Growth Conditions and Preparation of Crude Extracts—LB medium (29) supplemented with appropriate antibiotics (chloramphenicol (4 μg/ml) or spectinomycin (100 μg/ml) for *B. subtilis*, and ampicillin (50 μg/ml) for *E. coli*) was routinely used for selection of transformants. BL21(DE3) transformants were grown at 30 °C in 2 × YT medium (28) supplemented with 50 μg/ml ampicillin.

B. subtilis extracts for phosphorylation assays were prepared as follows. Cells were grown in liquid LB medium supplemented with sucrose (30 mM) and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) to allow *sacX* expression from the *spac* promoter carried by pSL151. Cells were harvested by centrifugation when the culture reached an A_{600} of 1.5 and resuspended in a *B. subtilis* lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). Cells were ruptured by sonication, and extracts were then centrifuged at $10,000 × g$ for 5 min. Aliquots of the supernatants were stored at −80 °C until used.

Protein Purification—Enzyme I and HPr were prepared as described previously (30, 31). The wild-type and mutant SacY proteins as well as the P1 and P2 domains were produced and purified as GST-fusions. For this purpose, BL21(DE3) was transformed with the pGEX-2T derivatives, and transformants were grown with shaking at 30 °C in 200 ml of 2 × YT liquid medium. Protein overproduction was induced by addition of IPTG (0.5 mM) at an A_{600} of 0.5. After an additional 4-h incubation at 30 °C, cells were harvested by centrifugation and resuspended in an *E. coli* lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 5 mM benzamidine). Cells were lysed by sonication, and membranes were removed by a 30-min centrifugation at $100,000 × g$. The supernatant was then applied to a prepacked glutathione-Sepharose 4*B* column (Pharmacia), and fusion proteins were purified as recommended by the manufacturer. Purity of the proteins was confirmed by electrophoresis on 12.5% SDS-polyacrylamide gels (32). Protein concentrations were determined by the Bradford method (33).

[³²P]PEP Synthesis and Protein Phosphorylation—Chemical synthesis of [³²P]PEP from [³²P]orthophosphate (ICN, Costa Mesa, CA) was performed as described previously (34). The reaction mixture was loaded onto an AG1 × 8 column (Bio-Rad), preequilibrated with 0.1 M ammonium bicarbonate, and [³²P]PEP was separated from [³²P]orthophosphate using 5-ml aliquots of 0.2, 0.4, and 0.7 M ammonium bicarbonate. Fractions of 1 ml were collected, and eluted material was identified by thin layer chromatography on silica plates and *tert*-amyl alcohol/formate/H₂O (3:2:1) as a solvent. Under these conditions, [³²P]orthophosphate eluted in fractions 7 and 8 while [³²P]PEP eluted in fractions 11–13. Purified SacY (2.8 μg, used as the GST-fusion or separated from GST after cleavage with thrombin) was phosphorylated by incubation at 37 °C in a reaction mixture (15 μl final volume) containing 50 mM potassium phosphate buffer (pH 7.4), 2 mM dithiothreitol, 0.5 mM MgCl₂, 2.5 μM (0.25 μCi) [³²P]PEP, and either *B. subtilis* crude extract (17 μg) or the purified PTS proteins EI (3 μg) and HPr (1 or 0.1 μg). The reaction was terminated after 20 min by addition of SDS-electrophoresis sample buffer. This incubation time allowed for maximal protein phosphorylation, as determined by kinetic experiments (up to 60 min). Proteins were separated on 12.5% SDS-polyacrylamide gels. Autoradiography of dried gels were performed with Kodak x-ray film (X-OMAT) with exposure times ranging between 2 and 24 h at −80 °C.

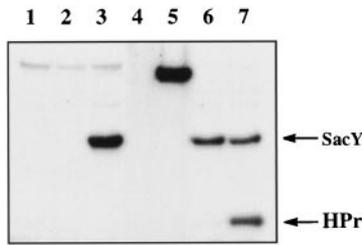*β-Galactosidase Assays*—*B. subtilis* liquid cultures were grown in

Multiple Phosphorylation of the SacY Antiterminator

FIG. 1. **SacY is phosphorylated by PEP in a PTS-dependent reaction.** Autoradiogram of an SDS-polyacrylamide gel showing the radiolabeled proteins obtained by incubation of 14 μg of a crude extract from GM1288X (*lanes 1–3*) with [^{32}P]PEP and 4 μg of the 55-amino acid N-terminal domain of SacY fused to the GST (*lane 2*) or 2.8 μg of the entire SacY protein (*lane 3*). 2.8 μg of the entire SacY protein was also incubated in the presence of [^{32}P]PEP and 14 μg of crude extract from GM1386X (*lane 4*) or GM1320X (*lanes 5–7*) supplemented with 0.1 or 1 μg of purified *B. subtilis* HPr (*lanes 6* and *7*, respectively). The *arrows* indicate the positions of SacY and HPr.

CgCH medium (7) supplemented with chloramphenicol (4 mg/l), tryptophan (50 mg/l), with or without 60 mM sucrose. Preparation of *B. subtilis* extracts and β-galactosidase assays were performed as described previously (29, 35). Assays were repeated with at least three independent transformants for each construct. The mean value is given for each assay. Standard deviations were not more than 15% for activities equal to or higher than 3.5 Miller units and not more than 40% for lower activities.

RESULTS

SacY Is Phosphorylated by HPr(His~P)—PTS-catalyzed phosphorylation of SacY was examined using *in vitro* phosphorylation assays. Purified SacY was incubated in the presence of [^{32}P]PEP and a crude extract prepared from *B. subtilis* GM1288X. Phosphorylation of the 55-amino acid N-terminal domain of SacY fused to GST was examined under the same conditions. As shown, SacY was readily phosphorylated by PEP (Fig. 1, *lane 3*), whereas no phosphorylation of the 55-amino acid N-terminal peptide was detected (Fig. 1, *lane 2*). The involvement of the general PTS enzymes in this phosphorylation was examined by incubating SacY in the presence of a crude extract from GM1320X (*ptsH*) or GM1386X (*ptsI*). No phosphorylation could be detected when SacY was incubated in the presence of a GM1386X crude extract (Fig. 1, *lane 4*). SacY was also not phosphorylated when incubated with a crude extract prepared from GM1320X (Fig. 1, *lane 5*). Nevertheless, SacY phosphorylation was observed following addition of 0.1 or 1 μg of purified HPr to extracts of the *ptsH* mutant (Fig. 1, *lanes 6* and *7*). These results demonstrate that SacY is phosphorylated in a PTS-dependent reaction involving the general energy coupling proteins, EI and HPr.

In the following phosphorylation experiments, the *B. subtilis* crude extract containing all the PTS components was replaced by the purified *B. subtilis* general energy coupling proteins EI and HPr. In these assays, EI, HPr, and SacY were incubated in all possible combinations in the presence of [^{32}P]PEP. As shown in Fig. 2, SacY was readily phosphorylated in the presence of both EI and HPr, whereas no SacY phosphorylation was detected when at least one of the two general PTS proteins was absent. Interestingly, SacX appeared dispensable although the extent of SacY phosphorylation was somewhat higher in the presence of the crude extract (Fig. 1, *lanes 6* and *7*) than in the presence of the purified EI and HPr (see Fig. 2). These results show that SacY can serve as phosphoryl acceptor with the general energy-coupling protein, HPr(His~P) serving as the phosphoryl donor, and that phosphorylation of SacY occurs outside of the first 55 N-terminal amino acids. The phosphate groups of phosphorylated SacY, like that of EI and HPr, were unstable under acidic conditions or during heat treatment (data not shown), suggesting that, as are EI and HPr, SacY is
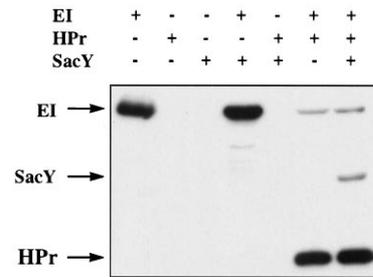
FIG. 2. **Direct phosphorylation of SacY by HPr(His~P).** Autoradiogram of an SDS-polyacrylamide gel showing the radiolabeled proteins obtained by incubation in the presence of [^{32}P]PEP, of purified EI (3 μg), HPr (1 μg), and SacY (2.8 μg), mixed in all combinations as indicated *above* the corresponding *lane*. The *arrows* indicate the position of each protein.

phosphorylated on histidyl residues. Studies described below were carried out to identify the phosphorylatable residues in SacY.

Sequence Analysis of Transcriptional Regulators Bearing Domains Conserved in the SacY Family—In silico analyses of SacY and its homologues were performed to derive guidelines for a biochemically based search of the phosphoryl acceptor residues in these proteins. Recent studies have identified the mRNA-binding domain of SacY as the N-terminal 55 amino acids of the antiterminator (36). Homologous N-terminal mRNA-binding domains are also present in six fully sequenced SacY homologues. Sequence analysis of these seven proteins revealed that their C-terminal regions consist of two homologous domains (~100 residues each), herein designated P1 (the N-terminal domain) and P2 (the C-terminal domain; see Fig. 3). P domains are also present in four partially sequenced putative transcriptional regulatory proteins, one in GlcR of *Staphylococcus carnosus*, one in LicT of *Bacillus amyloliquefaciens*, one in CasR of *Klebsiella oxytoca*, and two in CelR of *Bacillus stearothermophilus*. Two P domains are also present in each of three distinct transcriptional initiation regulatory proteins, the helix-turn-helix motif possessing DNA-binding LicR (previously named CelR; see Ref. 37), LevR of *B. subtilis*, and MtlR of *B. stearothermophilus* (see Fig. 3). Altogether, 25 homologous P domains have been identified in 14 modular transcriptional regulators. Significantly, homologous P domains could not be identified in eukaryotes or in bacteria lacking the PTS.

Binary comparison scores calculated for all P1 and P2 domains revealed that (i) all P domains are homologous, (ii) the P1 domains are more closely related to themselves than to the P2 domains, and (iii) the antiterminator P2 domains, but not the DNA binding protein P2 domains, are more closely related to themselves than to the P1 domains (data not shown). These findings lead us to propose that intragenic duplication of the gene encoding the ancestral P domain gave rise to the present day mRNA-binding antiterminators bearing two homologous P domains and that the P2 domains of the antiterminators evolved to serve a function distinct from that of the P1 domains. Surprisingly, all six P domains of the DNA-binding proteins (LevR, LicR, and MtlR) exhibit higher similarity with the P1 domains than with the P2 domains of the mRNA-binding antiterminators (data not shown; see below for interpretation).

The phylogenetic tree of all 25 sequenced P domains (Fig. 4) confirmed the conclusions derived from the binary statistical analysis. Although the branch lengths vary substantially, the tree is largely symmetrical with all P1 domains depicted on the left and most P2 domains (except those of LevR, LicR, and MtlR) depicted on the right. The tree reveals (i) that the antiterminator P1 domains comprise a tighter cluster than the corresponding cluster of the P2 domains, (ii) that the P1 and P2
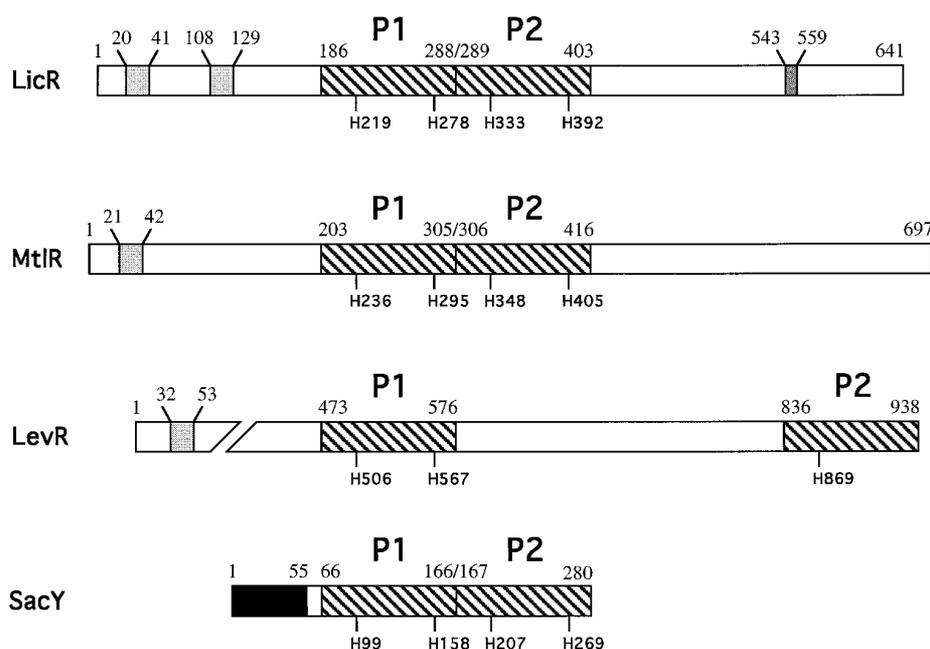
FIG. 3. **Schematic depiction of prototypical transcriptional regulators bearing two homologous P domains.** Shown are the helix-turn-helix bearing DNA-binding regulators LicR and LevR of *B. subtilis*, MtlR of *B. stearothermophilus*, and the RNA-binding antiterminator SacY of *B. subtilis*. The structural organization of SacY is similar to the domain structures and domain orders of SacT and LicT of *B. subtilis*, BglG of *E. coli*, ArbG of *Erwinia chrysanthemi*, AbgG of *Clostridium longisporum*, and BglR of *Lactococcus lactis*. A single P domain is also found in three partially sequenced putative regulatory proteins, *i.e.* GlcR of *S. carnosus*, LicT of *B. amyloliquefaciens*, and CasR of *K. oxytoca* (not shown). The putative limits of the indicated domains should not be considered as the precise boundaries. Also shown are the positions of the conserved histidines that were predicted to be phosphoryl acceptors (see text). The different portions or domains of the various proteins are indicated as follows: *black box*, mRNA-binding domain; *hatched boxes*, P1 and P2 domains; *light gray boxes*, N-terminal helix-turn helix motif; *dark gray box*, C-terminal motif conserved in IIA^{Fru}/IIA^{Mtl}/IIA^{Ntr} family of PTS proteins (38).

clusters of the antiterminators exhibit a near mirror image appearance, and (iii) that the P2 domains of the DNA-binding regulators also exhibit a near mirror image appearance, but they are more closely aligned with the antiterminator P1 domains. We propose that the antiterminator P2 domains evolved to assume a function dissimilar from that of the P1 domains while the P2 domains of the DNA-binding proteins did not.

When the 25 sequenced P1 and P2 domains were multiply aligned, only three residues in the alignment proved to be conserved in at least 23 positions (Fig. 5). These amino acids, highlighted in *black*, are (i) the histidyl residue at alignment position 8 that corresponds, respectively, to His-99 and His-207 in the P1 and P2 domains of SacY (conserved in all 25 P domains), (ii) the arginyl residue at alignment position 15 (conserved in 23 P domains), and (iii) the glutamyl residue at alignment position 63 (conserved in 24 P domains). An additional histidyl residue (alignment position 74 in Fig. 5) that corresponds to His-158 and His-269 in the P1 and P2 domains of SacY, respectively, is conserved in all P1 domains and in all but three of the P2 domains (those of BglR, BglG, and LevR). It is noteworthy, however, that a histidyl residue is present in two of these P2 domains close to alignment position 74, *i.e.* in position 82 of P2BglG and in position 81 of P2LevR (see Fig. 5). Additionally, we suspect that a frameshift error was inadvertently introduced in the C-terminal region of BglR. The "correct" sequence at this region contains histidyl, glutamyl, and arginyl residues that can be aligned with the corresponding conserved C-terminal residues of the P2 domains (data not shown).

Altogether, the data provide evidence for a modular organization of the mRNA-binding antiterminators and the DNA-binding transcriptional regulators and provide clues regarding their functional residues. The high degree of conservation of the four histidyl residues in the 25 P domains and the lability of the phosphorylated SacY under acidic conditions, as well as during heat treatment, prompted us to investigate the possi-

bility that one or more of these histidyls in SacY can serve as phosphoryl acceptor from HPr(His~P).

SacY Is Phosphorylated on at Least Two Distinct Sites—A point mutation, replacing the His-99 (CAT) codon with a Tyr (TAT) codon, was introduced in *sacY* and was cloned into the pGEX-2T protein overproduction vector. GST-SacY and GST-SacYH99Y were then purified and used in [^{32}P]PEP-dependent phosphorylation assays in the presence of a GM1288X crude extract. SacY, from which the GST module had been removed by cleavage with thrombin, was similarly examined for phosphorylation. As shown in Fig. 6, *lane 2*, the GST-SacY protein was readily phosphorylated albeit to a lower extent than SacY. It was, nevertheless, phosphorylated sufficiently to allow use of the GST-fusion protein instead of the thrombin-cleaved protein in further experiments. The GST-SacYH99Y mutant protein (Fig. 6, *lane 3*) was similarly phosphorylated although significantly less efficiently than the corresponding wild-type protein. This last result suggests either that SacY is phosphorylated on His-99 as well as an additional site or that His-99 is not phosphorylated but has an effect on the phosphorylation of SacY at a distinct site.

Since His-99 and His-207 have homologous environments, the residual phosphorylation of GST-SacYH99Y could be due to phosphorylation of His-207. This possibility was tested by incubating purified GST-SacYH2O7Y or GST-SacYH99Y/H2O7Y with the GM1288X extract in the presence of [^{32}P]PEP (Fig. 6, *lanes 4* and *5*). The H2O7Y substitution led to a marked decrease in SacY phosphorylation. Furthermore, only very slight phosphorylation of GST-SacYH99Y/H2O7Y was detected on overexposed films. These results suggest the presence of multiple phosphorylation sites in SacY.

Further experiments to reveal the putative phosphorylation sites of SacY were carried out with the purified regulatory P domains. The P1 and P2 domains of SacY (wild-type and the H99Y and H2O7Y mutants) were purified as GST fusions. Fig.

FIG. 4. **Phylogenetic tree of the 25 sequenced P1 and P2 domains.** Construction of the tree was as described by Reizer and Reizer (39) using the progressive multiple alignment method of Feng and Doolittle (40). Relative branch lengths (in arbitrary units) are provided adjacent to the branches. *Dashed branches* denote partially sequenced proteins bearing a single P domain. Abbreviations used and accession numbers (the prefix *P* and *L, U, M,* or *X* denote accession numbers for SwissProt and GenBank™/EBI data banks, respectively), are as follows: SacY(Bsu), the antiterminator of the *sacB* operon of *B. subtilis* (P15401); SacT(Bsu), the antiterminator of the *sacPA* operon of *B. subtilis* (P26212; note that the first 23 residues of SacT(Bsu) were deleted in M33761); LicT(Bsu), the antiterminator of the *licTS* and *bglPH* operons of *B. subtilis* (P39805); BglG(Eco), the antiterminator of the *bgl* operon of *E. coli* (P11989); ArbG(Ech), the antiterminator of the *arb* operon of *E. chrysanthemi* (P26211); BglR(Lla), the antiterminator of the *bgl* operon of *L. lactis* (L27422); AbgG(Clo), a putative antiterminator of the *abg* operon of *C. longisporum* (L49336); CasR(Kox), partially sequenced, putative regulatory protein of the *cas* operon of *K. oxytoca* (U61727); LicR(Bsu), helix-turn-helix bearing transcriptional regulator of the *licBCAH* operon of *B. subtilis* (P46321); CelR(Bst), partially sequenced regulatory protein of the *cel* operon of *B. stearothermophilus* (U07818); LevR(Bsu), DNA-binding transcriptional activator of the levanase operon of *B. subtilis* (P23914); MtlR(Bst), helix-turn-helix bearing transcriptional regulator of the *mtl* operon of *B. stearothermophilus* (U18943); LicT(Bam), partially sequenced protein of *B. amyloliquefaciens* (M15674); GlcR(Sca), partially sequenced, putative regulator of the operon encoding the twin glucose-specific enzymes II of *S. carnosus* (X93360). Not shown here and in Fig. 5 are the P domains of the recently sequenced antiterminator (LacT) of the *lac* operon of *Lactobacillus casei* (Z80834). The designations proposed here for the partially sequenced GlcR of *S. carnosus* and LicT of *B. amyloliquefaciens* is in accordance with the published designations of other protein members of this family.

7 shows the results obtained when the purified GST-P1, GST-P2, GST-P1H99Y, and GST-P2H2O7Y were incubated with the GM1288X extract in the presence of [³²P]PEP. GST-P1 (Fig. 7, *lane 3*) and GST-P2 (Fig. 7, *lane 4*) were both readily phosphorylated, showing that SacY can be phosphorylated on at least two distinct sites located within the P1 and P2 domains. The GST-P2H2O7Y domain was slightly phosphorylated (Fig. 7, *lane 6*), whereas no phosphorylation of the GST-P1H99Y domain was detected (Fig. 7, *lane 5*). Similar phosphorylation patterns were obtained when these domains were incubated with purified EI and HPr (data not shown). Taken together, these results strongly suggest that SacY is phosphorylated on His-99, His-207, and another residue located in P2. They further suggest that either His-158 is not phosphorylated or the H99Y mutation prevents phosphorylation of His-158. As the other conserved histidine in P2 is His-269, we purified the GST-P2H269Y and the GST-P2H2O7Y/H269Y mutant proteins and subjected them to phosphorylation assays in the presence of the *B. subtilis* GM1288X extract (Fig. 8*A*) or purified EI and HPr (Fig. 8*B*). As shown, the GST-P2H2O7Y and GST-P2H269Y mutant domains were both phosphorylated although much less efficiently than the corresponding wild-type P2 domain. Phosphorylation of the doubly mutated GST-P2H2O7Y/H269Y domain could not be detected.

These results provide evidence for multisite phosphorylation of SacY by HPr(His~P). They further suggest that phosphorylation occurs on P1 and P2, very likely on His-99, His-207, and His-269.

Effects of Substitution of the Conserved Histidines on SacY Antitermination Activity—The involvement of the four conserved histidines (His-99, His-158, His-207, and His-269) in the regulation of SacY antitermination activity was tested *in vivo*. Genes encoding proteins in which one, two, or three of these histidines were replaced with tyrosines were introduced into plasmid pSL90 carrying the *sacXY* coding sequences to integrate the different alleles at the chromosomal *sacY* locus. These pSL90 derivatives were used to transform strain GM152 carrying a *sacB'-lacZ* reporter fusion, a deletion of the 3' part of *sacX*, and a deletion of the entire *sacY* gene. As shown in Table I, the H99Y substitution led to strongly constitutive expression of *sacB'-lacZ*. The H269Y substitution slightly increased expression of the reporter gene, whereas the two other point mutations, H158Y and H2O7Y, did not significantly modify the phenotype when compared with wild-type SacY. The double mutants H99Y/H2O7Y and H99Y/H269Y, as well as the H99Y/H2O7Y/H269Y triple mutant exhibited strong constitutive expression of the fusion, comparable with that conferred by the single H99Y mutation. Increased expression conferred by

```
                            1                                                35
P1SacY(Bsu)   92   I H V A F S D H L S F A I E R L S N G . . . . M V I K N P L L N E I K
P1SacT(Bsu)   90   I H I A L S D H L S F A I E R I Q N G . . . . L L V Q N K L L H E I K
P1LicT(Bsu)   93   I Y V S L T D H I N F A I Q R N Q K G . . . . L D I K N A L L W E T K
P1AbgG(Clo)   94   I Y V V T L T D H I N Y V I E A C K Q G . . . . I S K P N I L I W E I K
P1BglR(Lla)   92   V L V A F A D H L N A A I I R E K D N . . . . I T I K N F L L W D I K
P1ArbG(Ech)   93   L Y I S L T D H C H F A I E R H R Q G . . . . V D I R N G L Q W E V K
P1BglG(Eco)   94   I Y I S L T D H C Q F A I K R F Q Q N . . . . V L L P N P L L W D I Q
P1CelR(Bst)  x+26  V M Y A L A M H I Q T A I N R L R A G . . . . T I V S H P K L N E V R
P1MtlR(Bst)  229   S Y I A L V V H L A L A I E R I S Q G . . . . E S I N F D Q Q Y L E T
P1LicR(Bsu)  212   G L N N L I I H I A I A C K R I R T E . . . . N Y V S L F P K D M D H
P1LevR(Bsu)  499   F I Y F L S M H I D A F L K R G K Q I . . . . D V L N T Q E T D E I R
P2SacY(Bsu)  200   S Y E R L V T H L R F A I Q H I K A G E . S I Y E L D A E M I D I I K
P2SacT(Bsu)  198   S Y Q R L V T H L R Y A V S R L E S N E . A L H R M D E E M L Y F I Q
P2LicT(Bsu)  200   H Y Y R F V T H L K F F A Q R L F N G T . H M E S Q D D F L L D T V K
P2AbgG(Clo)  204   N Y E R F I T H L R F F F K R L E R K E . I T E S E D N F L L E Q V K
P2BglR(Lla)  199   Y F Q R F I T H L K F F T E R I L S N T K S D E S T D E D L F L L I T
P2ArbG(Ech)  200   S Y H R F V T H L K F F A Q R L L G R T . P V F S E D E S L H D V V K
P2BglG(Eco)  201   S Y Q R L V T H L K F L S W R I L E H A . S I N D S D E S L Q Q A V K
P2CelR(Bst)  x+383 D L V G L V F H L C C L L D R L L S G E R T S G D R G A A K A C G H E
P2MtlR(Bst)  341   L Y E D L V V H L K P A L Y R I Q H N . . . . . M G I A N P L L E K I V
P2LicR(Bsu)  326   L K I G L A L H M K P A I S R N R Y G . . . . M N L R N P M L A A I K
P2LevR(Bsu)  862   V L I K V I M H T A F A F E R V I K Q . . . . N P I A F L E E E E I N
P2LicT(Bam)  x+8   H Y Y R F V T H L K F F D Q R L F N G T . H M E S Q D D F L L E T V K
P2CasR(Kox)  x+18  S Y Q R F V T H L K F F A Q R M L T R T . V V E D D D V T L H S A V K
P2GlcR(Sca)  x+56  Q Y Q R F I R H I Q F L I R R L Q K G E . . I I Q V N D E F G N M L K
Consensus          . Y . . . L . . H L . F A . . R . . . G . . . . . . . . . . L . . . . K

                                                                             70
P1SacY(Bsu)  124   L Y P K E F Q I G L W A R A L I K D K L G I H I P D D E I G N I A M .
P1SacT(Bsu)  122   L Y K K E Y E I G L W A I G H V K E T L G V S L P E D E A G Y I A L .
P1LicT(Bsu)  125   L Y K D E F A I G K E A L V M V K N K T G V S L P E D E A G F I A L .
P1AbgG(Clo)  126   F Y P K E F A V G L K A I E F I E D E L G Y K L H E D E A G N I A L .
P1BglR(Lla)  124   F F P E E F A L C L E T L Q K V Q E K L N I S L P E D E A G F L A M .
P1ArbG(Ech)  125   L Y Q K E F A I G L D A L D I I H R R L G V R L P E D E A G F I A L .
P1BglG(Eco)  126   L Y P K E F Q L G E E A L T I I D K R L G V Q L P K D E V G F I A M .
P1CelR(Bst)  x+58  A Y K Q E F A V A L D C L Q L M E E R T N I D F P I D E A G F L T M F
P1MtlR(Bst)  261   Q T T K E Y E T A E K I A R S L E H A F R I T I P K E E I G Y I T M .
P1LicR(Bsu)  244   L H Q K E Y T L A Y H C A E K I V K E L E S K L S V T F P K D E T A Y I T M .
P1LevR(Bsu)  531   T H V K E Y R V A M I F K D K I Q E Y F K V A I P E I E V I Y L T M .
P2SacY(Bsu)  235   K F K D A F L C A L S I G T F V K K E Y G F E F P E K E L C Y I A M .
P2SacT(Bsu)  233   K Y S F A Y Q C A L E L A E F L K N E Y Q L H L P E S E A G Y I T L .
P2LicT(Bsu)  235   K Y H R A Y E C T K K I Q T Y I E R E Y E H K L T S D E L L Y L T I .
P2AbgG(Clo)  239   K Y T D A Y E C M L K I E K Y L . . . . G Q E L S N E E Q L Y L M L .
P2BglR(Lla)  235   K Y P R A Y I G T K K S V S F L N R Q V R T K F . . . . . . . . . . L .
P2ArbG(Ech)  235   K Y T L A Y H C A E K I Q D H I M L H Y D Y T L T K E E I M F L A I .
P2BglG(Eco)  236   N Y P Q A W Q C A E R I A I F I G L Q Y Q R K I S P A E I M F L A I .
P2CelR(Bst)  x+419 E D G A L Y R A V K E G L F P L E Q Q Y G V C I D E D E L C H I V . .
P2MtlR(Bst)  373   D Y P E L F . . A V L E K G V K Q V F P D V T V P K E E I G Y L V L .
P2LicR(Bsu)  358   H Y P L A F E A G I I A G I V I K E Q T G I E I H E N E I G Y L A L .
P2LevR(Bsu)  894   Q L K E M V Y V T E R T L A P Y E E K L G L R I S D D E K L F I A . .
P2LicT(Bam)  x+43  K Y H Q A Y K C T K N I H T Y I E K E Y G H K L T S D E L L Y L T I .
P2CasR(Kox)  x+53  N Y P K A W K C A E T V A R H L Q K S Y Q R P L T T E E I M F L A I .
P2GlcR(Sca)  x+90  H Y P L C Y N I A V K I I K M M Q Q H L D E V V Y E A E L I Y L T L .
Consensus          . Y . . . . . . . . . . . . . . . . . . . . . . . . . P . D E . . . . A . . .

                                       83
P1SacY(Bsu)  158   . . . H I H T A R . N N A
P1SacT(Bsu)  156   . . . H I H T A K . M D A
P1LicT(Bsu)  159   . . . H I V N A E . L N E
P1AbgG(Clo)  160   . . . H L I N A Q . V N E
P1BglR(Lla)  158   . . . H I V N G T . L G S
P1ArbG(Ech)  159   . . . H L V N A Q . L D S
P1BglG(Eco)  160   . . . H L V S A Q . M S G
P1CelR(Bst)  x+93  F A F H E E Q A E . E R E
P1MtlR(Bst)  295   . . . H L M G A K . L R D
P1LicR(Bsu)  278   . . . H L L G T K R M T Q
P1LevR(Bsu)  565   . L I H . . S I K S L K E
P2SacY(Bsu)  269   . . . H I Q R F Y Q R S V
P2SacT(Bsu)  267   . . . H V Q R L . . Q D L
P2LicT(Bsu)  269   . . . H I E R V V K Q A .
P2AbgG(Clo)  269   . . . H I Q R V T T R E K
P2BglR(Lla)  260   . . . K M D K Y I . . . .
P2ArbG(Ech)  269   . . . H I E R V R S E L Q
P2BglG(Eco)  270   . . . N I E R V R K E H .
P2CelR(Bst)  x+452 . . . H F F R S L Q G K R
P2MtlR(Bst)  405   . . . H F A A A L L R E K
P2LicR(Bsu)  392   . . . H F G A A I E R K K
P2LevR(Bsu)  927   . . . A I F A E E V H G Q
P2LicT(Bam)  x+77  . . . H I E R V V K Q V .
P2CasR(Kox)  x+87  . . . H I E R V R K E G R
P2GlcR(Sca)  x+124 . . . H I N H F T Q Q N .
Consensus          H I . . . . . . . . .
```

FIG. 5. **Multiple alignment of 25 sequenced P1 and P2 domains.** The alignment shows the conserved regions of 25 P1 and P2 domains present in the seven currently sequenced mRNA-binding transcriptional antiterminators of the SacY/BglG family (SacY(Bsu), SacT(Bsu), BglG-(Eco), AbgG(Clo), LicT(Bsu), ArbG(Ech) and BglR(LLa)), three helix-turn-helix bearing DNA-binding proteins (LevR(Bsu), LicR(Bsu) and MtlR(Bst)), and four partially sequenced, putative transcriptional regulators (GlcR(Sca), LicT(Bam), CelR-(Bst), and CasR(Kox)). The alignment was generated using the TREE program of Feng and Doolittle (40) and adjusted manually at alignment positions 70–75 of the P2 domains. The *horizontal line* segregates the aligned P1 domains from their aligned P2 counterparts. *Numbers* at the *top* of the aligned sequences denote the residue positions in the multiple alignment. The residue number in each protein is provided at the beginning of each line. *X* indicates uncertainty of the residue number due to unavailability of the N-terminal sequence. Positions that are fully conserved or that are conserved with only one or two exceptions are *highlighted in black*. *Boxed residues* in either the P1 or P2 domains are fully conserved or conserved with only 1–3 exceptions. The consensus sequence (at least 13 residues conserved) is provided below the multiple alignment. Abbreviations for the various proteins from which the P domains were derived are as indicated in the legend of Fig. 4. Precise limits of P1 and P2 domains can be provided upon request.

H269Y was further slightly enhanced by addition of the H158Y mutation, whereas addition of H2O7Y or H158Y/H2O7Y had no significant effect when compared with the H269Y single mutation. Thus, while H99Y mutation conferred strong constitutive expression of *sacB* when present alone or in combination with other mutations, H269Y had only a slight positive effect. These results indicate that His-99 is the major target of the regulation of SacY antitermination activity.

DISCUSSION

In silico, in vitro, and *in vivo* analyses described here provide evidence for the modular organization of SacY, PTS-catalyzed phosphorylation of its duplicated P1 and P2 domains, and the phosphorylation-dependent regulation of SacY antitermination activity. The data strongly suggest that His-99, His-207, and His-269 in SacY can be phosphorylated by HPr(His~P) since (i) substitution of residues 99 and/or 207 drastically decreased

phosphorylation of the complete SacY protein, (ii) substitution of His-99 in the purified P1 domain abolished phosphorylation of this domain, and (iii) substitution of His-207 or His-269 of P2 led to strongly decreased phosphorylation of this domain while the double mutant GST-P2H2O7Y/H269Y was not phosphorylated. Since His-99, His-207, and His-269 are highly conserved in homologous RNA-binding antiterminators as well as in functionally distinct DNA-binding transcriptional regulators (see Fig. 5), we propose that PTS-catalyzed phosphorylation of the conserved histidines plays a role in the regulation of transcription by proteins possessing P domains. Thus, in these transcriptional regulators, phosphorylation of the P domains would be involved in the transduction of signal (presence/absence of a sugar) to the effector domain (*i.e.* the RNA- or DNA-binding domain).

LicT, a SacY homologous antiterminator, has recently been shown to be phosphorylated at multiple sites; separation of proteolytic fragments, obtained from phosphorylated ^{32}P-labeled LicT, showed that LicT is phosphorylated by HPr(His~P) on four histidines corresponding to His-99, His-158, His-207,
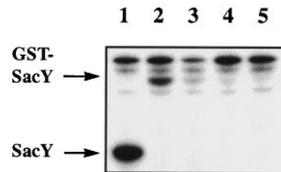
FIG. 6. **Substitution of His-99 and/or His-207 in SacY affects its phosphorylation.** Autoradiogram of an SDS-polyacrylamide gel showing the radiolabeled proteins obtained by incubation of 14 μg of crude extract from GM1288X in the presence of 2.8 μg of SacY (*lane 1*), GST-SacY (*lane 2*), GST-SacYH99Y (*lane 3*), GST-SacYH2O7Y (*lane 4*), or GST-SacYH99Y/H2O7Y (*lane 5*) and [^{32}P]PEP. The *arrows* indicate the positions of the SacY and GST-SacY proteins.
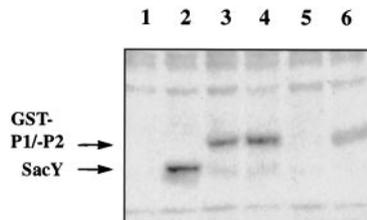
FIG. 7. **SacY is phosphorylated on both P1 and P2 domains.** Autoradiogram of an SDS-polyacrylamide gel showing the radiolabeled proteins obtained by incubation, in the presence of [^{32}P]PEP, of 14 μg of crude extract from GM1288X without addition (*lane 1*) or with addition of 2.8 μg of SacY (*lane 2*), 17 μg of GST-P1 (*lane 3*), or GST-P2 (*lane 4*), 34 μg of GST-P1H99Y (*lane 5*), or GST-P2H2O7Y (*lane 6*). The *arrows* indicate the positions of the SacY and GST-P proteins.

and His-269 of SacY[2] While the reported findings using the recombinant P1 and P2 domains of SacY clearly indicate that His-99, His-207, and His-269 are phosphorylated by HPr(His~P), we cannot eliminate the possibility that His-158 is phosphorylated in the complete protein. The occurrence of multisite phosphorylation of LicT is not surprising since its antitermination activity is modulated both positively and negatively by the PTS (22). However, multisite phosphorylation of SacY was unexpected since this regulator is only subject to a negative control by the PTS (7).

Previous genetic studies had already suggested that His-99 phosphorylation in SacY negatively regulates antitermination activity (7). Our results show that H99Y mutation, introduced alone or in combination with substitutions of other conserved histidines, confers a strong constitutive expression of *sacB*. This dominant constitutive phenotype indicates that His-99 is the major determinant for the control of SacY antitermination activity. Phosphorylation of one (or two) of the other conserved histidines in SacY could play an indirect role, by modulating the level of His-99 phosphorylation. Indeed, the positive effect conferred by the H269Y single mutation, the H158Y/H269Y double mutation and the H158Y/H2O7Y/H269Y triple mutation on *sacB* expression suggests the possibility of phosphotransfer from His-269 to His-99 in the wild type protein, albeit *in vitro* experiments clearly showed that His-99 could be directly phosphorylated by HPr(His~P). Alternatively, phosphorylation of other histidyl residues in SacY might correspond only to the positive regulation exerted by the PTS on SacT and LicT. This suggestion stems from the isolation of *sacT* and *licT* mutants possessing antitermination activity that is independent of EI and HPr.[3] However, one of these LicT mutant proteins has been shown to be phosphorylated *in vitro* on the same histidines and to the same extent as the wild-type protein.[3] Additionally, our *in vitro* assays clearly showed that the H2O7Y substitution severely reduced overall phosphorylation of SacY (Fig. 6, *lane 4*). Similarly, phosphorylation experiments with LicT, carried out under similar conditions, revealed that His-207 was 4–10-fold more strongly phosphorylated as compared with the other phosphorylatable histidyl residues.[3] Finally, substitution of either histidine 99, 207, or 269 of SacY led to a markedly reduced phosphorylation of the complete protein, indicating that the effects of single substitutions are not additive. Since the phenotypes conferred *in vivo* by the different mutations (*e.g.* H2O7Y, Table I) do not always correlate with the level of SacY phosphorylation observed *in vitro* (Fig. 6, *lane 4*), it is conceivable that the relative phosphorylation level of each phosphorylatable histidine (and particularly His-99) is not equivalent under both conditions. Our *in vitro* phosphoryl-

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[2] C. Lindner, unpublished results.
[3] D. Le Coq and C. Lindner, unpublished results.
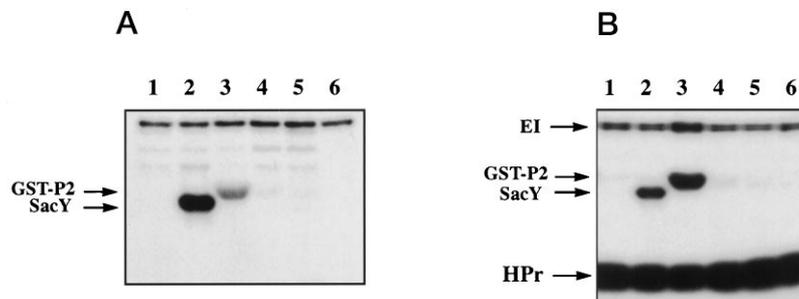
A

B

FIG. 8. **Effect of substitutions of histidines 207 and/or 269 on the phosphorylation of P2.** Autoradiogram of an SDS-polyacrylamide gel showing the radiolabeled proteins obtained by incubation, in the presence of [^{32}P]PEP, of 2.8 μg of SacY (*lane 2*), 17 μg of GST-P2 (*lane 3*), GST-P2H2O7Y (*lane 4*), GST-P2H269Y (*lane 5*), or GST-P2H2O7Y/H269Y (*lane 6*) with 14 μg of GM1288X crude extract (*panel A*) or 3 μg of EI and 1 μg of HPr (*panel B*). *Lane 1*, control (no addition of SacY). The *arrows* indicate the positions of the SacY and GST-P2 proteins.

TABLE I
Effect of different sacY alleles on sacB expression

SacY	*sacB'-lacZ* expression[a]	
	−Sucrose	+Sucrose
Wild-type	<0.1	3.3
H99Y	24.2	28.5
H158Y	<0.1	5.5
H207Y	<0.1	2.7
H269Y	1.0	9.8
H99Y,H207Y	24.3	30.4
H99Y,H269Y	42.3	30.2
H158Y,H207Y	<0.1	2.9
H158Y,H269Y	3.4	19.3
H207Y,H269Y	0.8	7.5
H99Y,H207Y,H269Y	25.6	27.0
H158Y,H207Y,H269Y	0.6	8.0

[a] Miller units. Values are the average of at least three experiments, performed with three independent transformants.

ation assays revealed multiple phosphorylation of SacY, and the *in vivo* experiments pointed out the critical role of His-99. Taken together, these results demonstrate that the antitermination activity of SacY is primarily controlled by PTS-dependent phosphorylation of His-99.

Unexpectedly, our *in vitro* assays showed that phosphorylation of SacY does not require the presence of SacX. This finding is not in agreement with the model proposed for the regulation of BglG, in which phosphorylated EIIBCA (BglF) transfers phosphate to the antiterminator (11, 13). On the other hand, direct phosphorylation of the homologous SacT by HPr(His~P) has been reported recently and was proposed to be one element of the positive control exerted by the PTS on SacT-dependent antitermination (4). The present data clearly demonstrate a link between phosphorylation of His-99 and the negative control exerted by the PTS on SacY activity. Furthermore, we show that HPr(His~P) can directly phosphorylate His-99 in the absence of any other sugar-specific PTS component. Since previous genetic data suggested the involvement of SacX as a negative regulator in *sacB* induction (7), we propose that the kinetics of SacY phosphorylation by HPr(His~P) is modified by the formation of a SacX/SacY complex. In the absence of sucrose, SacX would be phosphorylated by the PTS general enzymes *via* an unknown EIIA. Phosphorylated SacX could then trap SacY, allowing its efficient phosphorylation at His-99 by HPr(His~P). Thus, in the absence of sucrose, SacX might exert double negative control over antitermination activity by trapping SacY and by increasing its phosphorylation rate. During the induction response, SacX would be dephosphorylated by sucrose, thereby preventing its interaction with SacY, which in turn would be less efficiently phosphorylated at His-99. According to this proposal, the phosphorylation of SacY, which was observed *in vitro* in the absence of SacX, could result from the high concentrations of SacY and the PTS general enzymes used. In support of this model, we note that HPr(His~P) transfers its phosphoryl group to SacY more efficiently in the presence of crude extract, *i.e.* in the presence of SacX (Fig. 1), than in the presence of pure EI (Fig. 2). It is noteworthy that, although overproduced on a replicative plasmid, SacX was present in the crude extracts at much lower amounts than the SacY protein added in our assays.

This model will need to be substantiated by complementary biochemical studies. Effects of the addition of purified SacX on

SacY phosphorylation by HPr(His~P) will need to be measured, in the absence or presence of sucrose. In the same way, the occurrence of the SacX/SacY complex and its possible stabilization by addition of HPr(His~P) must be demonstrated. These studies are currently in progress.

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