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Phosphorylation and Functional Properties of the IIA Domain of the Lactose Transport Protein of *Streptococcus thermophilus*

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The lactose-H^+ symport protein (LacS) of *Streptococcus thermophilus* has a carboxyl-terminal regulatory domain (IIA^{LacS}) that is homologous to a family of proteins and protein domains of the phosphoenolpyruvate: carbohydrate phosphotransferase system (PTS) in various organisms, of which IIA^{Glc} of *Escherichia coli* is the best-characterized member. On the basis of these similarities, it was anticipated that IIA^{LacS} would be able to perform one or more functions associated with IIA^{Glc}, i.e., carry out phosphoryl transfer and/or affect other catabolic functions. The gene fragment encoding IIA^{LacS} was overexpressed in *Escherichia coli*, and the protein was purified in two steps by metal affinity and anion-exchange chromatography. IIA^{LacS} was unable to restore glucose uptake in a IIA^{Glc}-deficient strain, which is consistent with a very low rate of phosphorylation of IIA^{LacS} by phosphorylated HPr (HPr∼P) from *E. coli*. With HPr∼P from *S. thermophilus*, the rate was more than 10-fold higher, but the rate constants for the phosphorylation of IIA^{LacS} ($k_1 = 4.3 \times 10^2\ M^{-1}\ s^{-1}$) and dephosphorylation of IIA^{LacS}∼P by HPr ($k_{-1} = 1.1 \times 10^3\ M^{-1}\ s^{-1}$) are still at least 4 orders of magnitude lower than for the phosphoryltransfer between IIA^{Glc} and HPr from *E. coli*. This finding suggests that IIA^{LacS} has evolved into a protein domain whose main function is not to transfer phosphoryl groups rapidly. On the basis of sequence alignment of IIA proteins with and without putative phosphoryl transfer functions and the known structure of IIA^{Glc}, we constructed a double mutant [IIA^{LacS}(I548E/G556D)] that was predicted to have increased phosphoryl transfer activity. Indeed, the phosphorylation rate of IIA^{LacS}(I548E/G556D) by HPr∼P increased ($k_1 = 4.0 \times 10^3\ M^{-1}\ s^{-1}$) and became nearly independent of the source of HPr∼P (*S. thermophilus*, *Bacillus subtilis*, or *E. coli*). The increased phosphoryl transfer rate of IIA^{LacS}(I548E/G556D) was insufficient to complement IIA^{Glc} in PTS-mediated glucose transport in *E. coli*. Both IIA^{LacS} and IIA^{LacS}(I548E/G556D) could replace IIA^{Glc}, but in another function: they inhibited glycerol kinase (inducer exclusion) when present in the unphosphorylated form.

The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) catalyzes the uptake of carbohydrate concomitant with its phosphorylation. The phosphoryl group is transferred from PEP via the general energy-coupling proteins enzyme I and HPr to the sugar-specific phosphoryl transfer protein/domain IIA; phosphorylated IIA (IIA∼P) transfers the phosphoryl group to the sugar-specific IIB protein domain which phosphorylates the sugar that is translocated via the sugar-specific IIC protein domain (23). Apart from its function in the uptake and phosphorylation of sugars, the PTS regulates transport and subsequent metabolism of non-PTS carbohydrates. In gram-negative enteric bacteria, this regulation is mediated by the phosphorylation state of the Glc-specific IIA (IIA^{Glc}), which is determined by the relative rates of phosphorylation by HPr∼P and dephosphorylation by $IICB^{Glc}$. For instance, IIA^{Glc}∼P is involved in the stimulation of adenylate cyclase, whereby the expression of many catabolic enzymes is regulated through changes in cyclic AMP (cAMP) levels (1).

Unphosphorylated IIA^{Glc}, on the other hand, binds directly to several transporters and enzymes of carbohydrate metabolism, and thereby inhibits their activities, via a phenomenon called inducer exclusion (23). The interaction of IIA^{Glc} with one of its targets, glycerol kinase (GlpK), has been elucidated by analyzing the crystal structure of *Escherichia coli* glycerol kinase in complex with *E. coli* IIA^{Glc} (7). This study revealed that IIA^{Glc} binds to glycerol kinase at a region that is distant from the catalytic site of glycerol kinase, which suggests that long-range conformational changes mediate the inhibition of glycerol kinase by IIA^{Glc}.

In gram-positive bacteria, not only can HPr be phosphorylated by PEP/enzyme I on a histidine residue (His-15), but also a metabolite-activated ATP-dependent protein kinase can phosphorylate a serine residue at position 46 (2). The serine-phosphorylated form of HPr [HPr(Ser-P)] seems to control carbohydrate metabolism both at the protein level and at the gene level, i.e., transport activities (inducer exclusion of both PTS and non-PTS sugars and/or inducer expulsion) and transcription (28).

There is no evidence for the involvement of IIA^{Glc} or IIA-like proteins in PTS-mediated regulation in gram-positive bacteria. However, several non-PTS sugar transporters have a carboxyl-terminal domain that is homologous to IIA^{Glc} of *E. coli* (18, 20). The best-characterized system of this family of transporters with a two-domain structure is the lactose transport protein (LacS) of *Streptococcus thermophilus*. This protein is, among others, also homologous to the melibiose transport proteins of *Salmonella typhimurium* and *E. coli*, which lack a IIA-like domain but are regulated by IIA^{Glc} (18, 41). The carboxyl-terminal IIA domain of the LacS protein of *S. thermophilus* (IIA^{LacS}) is located in the cytoplasm and has 34% residue identity with IIA^{Glc} of *E. coli* (20). IIA^{LacS} is phosphorylated, most likely at His-552, by HPr∼P, which inhibits

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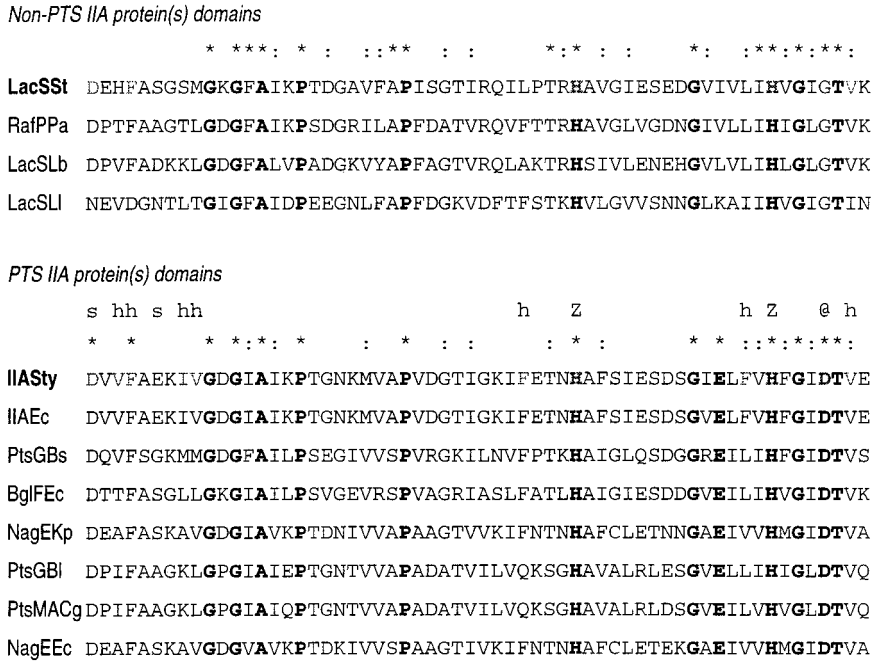

FIG. 1. Alignment of the active site regions of PTS IIA and non-PTS IIA protein domains. *, conserved residue; :, similar residue; s, salt bridge with residue in glycerol kinase; h, hydrophobic interaction with glycerol kinase; z, coordination of Zn(II) (7); @, charged group near phosphorylation site. Only a portion of each PTS IIA protein domain in the SwissProt database is shown. Two-letter suffixes denote *S. thermophilus* (St), *Pseudomonas aeruginosa* (Pa), *Lactobacillus bulgaricus* (Lb), *Leuconastoc lactis*, (Ll), *S. typhimurium* (Sty), *E. coli* (Ec), *B. subtilis* (Bs), *Klebsiella pneumoniae* (Kp), *Brevibacterium lactofermentum* (Bl), and *Corynebacterium glutamicum* (Cg).

the transport activity of LacS (17, 19). This histidine residue corresponds with His-90 of IIA^{Glc} in *E. coli*, which has been shown to be the phosphoryl-accepting site (4, 24) (Fig. 1). The phosphorylation of LacS by HPr~P has been assessed only qualitatively, and it is not known whether the IIA^{LacS} domain has phosphoryl transfer activity equivalent to that of IIA^{Glc}.

In this study, we investigated (i) the kinetics of (de)phosphorylation of the IIA^{LacS} domain of *S. thermophilus* and (ii) the ability of the protein to carry out phosphoryl transfer to IICB^{Glc} of *E. coli* (PTS-mediated glucose transport) and to inhibit glycerol kinase. Information about the (de)phosphorylation kinetics is relevant because the only known function of the IIA domain in LacS, and homologous transporters, involves the regulation of lactose-H$^+$ symport activity, for which a very rapid phosphoryl transfer may not be critical. For this study, we expressed IIA^{LacS} in *E. coli* and *S. typhimurium* and constructed two mutants in which either the proposed phosphoryl-accepting histidine was replaced by Arg [IIA^{LacS}(H552R)] or two residues near the putative phosphorylation site were replaced by the equivalent residues conserved in all PTS members of the IIA^{Glc} family [IIA^{LacS}(I548E/G556D)] (Fig. 1). The crystal structures of IIA^{Glc} from *E. coli* and *B. subtilis* show that the Glu residue is exposed to the surface of the molecule and may be critical for the interaction of IIA with its partner molecules; the Asp residue is close to the active site in the tertiary structure (12, 40). For the in vitro phosphorylation assays, we purified each of the mutant proteins and assessed phosphorylation by HPr~P from *E. coli*, *B. subtilis*, and *S. thermophilus*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used were *E. coli* DH5α [*deoR endA1 gyrA961 hsdR17* (r_K^- m_K^+) *recA rel-1 supE44 thi-1* Δ(*lacZYA*-arg-169) Ø80Δ*lacZ*Δλ] (8), BL21 [*hsdS gal*(λ *cIts*857) *ind-1 sam-7 nin-5 lacUV5*-T7 gene1] (32), LM1 [*crr-1 thi-1 his-1 argG6 metB galT rpsL ptsM manI nagE*] (11), and MC1061 [Δ(*lacIPOZYA*) F$^-$ *araD139* Δ(*ara-leu*)*7697 galU galK* r_K^+ m_K^- *strA*] (8) and *S. typhimurium* PP2178 [*crr-307*::Tn*10 nagE142 trpB223*] (36). To isolate plasmid DNA, the cells were grown in Luria broth under vigorous aeration at 37°C (29). For the transport assays, the cells were grown in minimal salts medium A (31) with glycerol (0.2% [wt/vol]) as carbon and energy source as described by van der Vlag et al. (37). For large-scale protein purification, the cells were grown in Luria broth in a 10-liter fermentor (Bio Bench ADI 1065; Applicon, Inc.) with oxygen supply (50% saturation) and pH control (pH 7.0). Growth on succinate was performed on agar plates containing minimal salts medium A supplemented with succinate (0.5%) and the essential nutrients as indicated by the autotrophic markers. When necessary, carbenicillin (50 μg/ml), chloramphenicol (10 μg/ml), tetracycline (12.5 μg/ml), or isopropyl-β-D-thiogalactopyranoside (1 mM) was added to the medium.

S. thermophilus ST11 (Δ*lacS*)/pGKHis was grown semianaerobically at 42°C in Belliker broth supplemented with 0.5% beef extract, 20 mM lactose, and 5 mg of erythromycin per ml (9).

DNA manipulations. DNA modifications were performed as described by Sambrook et al. (29) unless indicated otherwise. Subcloning of plasmids into *E. coli* strains was performed via *E. coli* DH5α. Plasmids used for the expression of proteins in *E. coli* and *S. typhimurium* are listed in Table 1.

Since alignments of IIA^{LacS} with other IIA proteins do not clearly reveal an optimal translation initiation site, several gene fragments specifying IIA^{LacS} were cloned. ATG initiation codons were engineered at positions 1359, 1383, and 1440 of the *lacS* gene, using the forward primers ARH (5′AGGAGGTGTCAACATGGCACGTCACGCTAAAATTGT), ELE (5′AGGAGGTGTCAACATGGAATTGGAACATCGCTTTAG), and VSL (5′AGGAGGTGTCAACATGGTATCTCTTGTAACCCCTAC), respectively; the corresponding protein domains are 181, 173, and 154 amino acids long. For the PCRs, use of the reverse primer BR (5′CAAAATACTTAGGATCCGAGTGAGCATC) generated a new *Bam*HI site 82 bp downstream of the stop codon of the *lacS* gene. For the PCRs with pSKE8e as the template DNA, the oligonucleotide primers were treated with T4 polynucleotide kinase before use. PCR fragments were isolated with QIA quick spin columns (Qiagen, Inc.) and then blunt-end ligated into pSKII$^-$ that had been digested with *Sma*I and dephosphorylated by Klenow enzyme. In the resulting plasmids pSK181, pSK173, and pSK154, the gene fragments encoding IIA^{LacS} are under the control of the T7 promoter. For the expression of IIA^{LacS} from the *tac* promoter, plasmid pKKELE was constructed by ligating the PCR ELE/BR fragment into pKK223-3 that had been linearized with *Sma*I and treated with Klenow enzyme.

For the expression of IIA^{LacS} from the *lacS* promoter and its native ribosome binding site and to generate a six-histidine tag at the C terminus of IIA^{LacS}, the *Nco*I/*Bam*HI fragment of pSKE8N was replaced by the NE/BR PCR fragment that had been treated with *Nco*I and *Bam*HI. The NE/BR PCR fragment was synthesized by using the oligonucleotide primer NE (5′GTCACCATGGAATTGGAACATCGC) as the forward primer, which introduced a new *Nco*I restric-

TABLE 1. Plasmids used

Plasmid	Properties[a]	Source or reference
pSKII$^-$	Amp^r, high-copy-number expression vector, T7 promoter	Stratagene
pSKE8	pSKII$^+$, carrying *lacS* of *S. thermophilus* A147 as 4,073-bp *Eco*RI fragment (*galM*$^+$ *lacS*$^+$)	19
pSKE8E	pSKE8 with additional *Eco*RI site 21 bp upstream of the initiation codon of *lacS*	9
pSKE8his	pSKE8 with His-tagged *lacS*	9
pSKE8N	pSKE8 with *Nco*I site at the initiation codon of *lacS*	9
pSKE8(*lac*S-H552R)	pSKE8 with His-552 of LacS replaced by Arg	19
pSKE8(*lac*S-I548E/G556D)	pSKE8 with Ile-548 and Gly-556 of LacS replaced by Glu and Asp, respectively	19
pSKoppAChis	pSKE8his with *lacS* (1,936-bp *Nco*I-*Mlu*I fragment) replaced by *oppA*	16a
pKK223-3	Amp^r, medium-copy-number expression vector, *taq* promoter	Pharmacia
pSK181	pSKII$^-$, carrying a 543 bp fragment[b] that specifies the C-terminal 181 amino acids of LacS	This work
pSK173	pSKII$^-$, carrying a 519-bp fragment[b] that specifies the C-terminal 173 amino acids of LacS	This work
pSK154	pSKII$^-$, carrying a 462-bp fragment[b] that specifies the C-terminal 154 amino acids of LacS	This work
pKKELE	pKK223-3, carrying a 631-bp fragment[b] that specifies IIA^{LacS}	This work
pSKIIAhis	pSKE8N with the 2,243-bp *Nco*I-*Bam*HI fragment replaced by a 644-bp *Nco*I-*Bam*HI fragment that specifies IIA$^{LacS-6H}$	This work
pSKIIAm1his	pSKIIAhis with H552R	This work
pSKIIAm2his	pSKIIAhis with I548E/G556D	This work
pGKHis	pGK13, carrying His-Tagged *lacS* of *S. thermophilus* as a 3,824-bp *Eco*RI-*Dra*I fragment from pSKE8 ligated into the *Eco*RI-*Eco*RV sites	9

[a] Amp and Cm indicate resistance to ampicillin and chloramphenicol, respectively.
[b] PCR fragment blunt-end ligated in the *Sma*I site of the vector.

tion site at the ATG initiation codon of IIA^{LacS}, BR as the reverse primer, and pSKE8his as the template DNA. IIA^{LacS} with mutations H552R and I548E/G556D was constructed by using pSKE8(*lac*S-H552R) and pSKE8(*lac*S-I548E/G556D), respectively, as template DNAs for the PCRs. NE and LBR (5′ CGC GGATCCTTTTTTGAAGGTAAT) were used as forward and reverse primers, respectively; LBR created a *Bam*HI restriction site 1 bp upstream of the stop codon of the *lacS* gene. After isolation of the PCR fragments and digestion with *Nco*I and *Bam*HI, the fragments were ligated into vector pSKoppAChis that had been treated with the corresponding enzymes. In this way, the gene fragments specifying mutant IIA^{LacS} were put under control of the *lacS* promoter, and the corresponding proteins had a six-histidine tag at the carboxyl terminus. All plasmids constructed were checked by restriction analysis and nucleotide sequencing using the Vistra automated laser fluorescent DNA sequencer system with the labeled primer cycle sequencing kit (Amersham, Inc.).

Nomenclature. IIA^{LacS} refers to the IIA domain of LacS in general, whereas constructs representing protein fragments of specific lengths are indicated by numbers between brackets (e.g., IIA^{LacS}[181] denotes a domain of 181 residues). The His-tagged IIA^{LacS}[173] protein, which was used in most of the experiments, is referred to as IIA$^{LacS-6H}$; when appropriate, mutations are indicated between parentheses [e.g., IIA$^{LacS-6H}$(H552R) and IIA$^{LacS-6H}$(I548E/G556D)]. Ile-548, His-552, and Gly-556 denote residue positions in LacS; the same numbering is used to indicate these positions in IIA^{LacS}.

Protein purification. All purification procedures were carried out at 4°C unless indicated otherwise. Enzyme I and HPr of *B. subtilis* and *E. coli* were purified as described previously (26, 27, 38)). For the isolation and purification of HPr from *S. thermophilus*, the cells were lysed after lysozyme treatment (6). For the removal of lysozyme, the supernatant of the cell lysate fraction was diluted 1× in Milli Q water and incubated with S-Sepharose (10 ml/g of lysozyme) for 1 h at 4°C. Fresh S-Sepharose was added twice, after removal of old resin by decanting the supernatant, after which the cell lysate fraction was incubated for another hour at 4°C. The proteins were then precipitated by addition of ammonium sulfate to 80% (wt/vol), and incubation overnight on ice water. After centrifugation (45 min at 70,000 × *g*), the pellet was dissolved in 20 mM Tris-HCl (pH 8.5) and loaded on a DEAE-Sepharose fast flow column (1.6 by 40 cm; Pharmacia Biotech Inc.) that had been equilibrated with 20 mM Tris-HCl (pH 8.5). The column was washed with 10 column volumes of 20 mM Tris-HCl (pH 7.0). Proteins were eluted with 10 column volumes of 20 mM Tris-HCl (pH 7.0)–40 mM NaCl and precipitated by 80% ammonium sulfate as described above. The pellet was dissolved in 20 mM sodium acetate (pH 4.0) and desalted on a PD-10 column (Pharmacia Biotech). The resulting fraction was loaded onto an S-Sepharose fast flow column (HR 5/5; Pharmacia Biotech) that had been equilibrated with 20 mM sodium acetate (pH 4.0). The proteins were eluted with a 250-ml gradient of 0 to 250 mM NaCl in 20 mM sodium acetate (pH 4.0). The fractions containing HPr were pooled and concentrated by 80% ammonium sulfate as described above. The pellet was dissolved in 50 mM potassium phosphate (KP_i; pH 7.0) to a concentration of 2 mg/ml.

For the isolation and purification of IIA^{LacS}, *E. coli* cells expressing IIA$^{LacS-6H}$ were grown to late exponential phase and harvested by centrifugation. The cells were washed twice with 50 mM KP_i (pH 8.0) and resuspended in buffer A (50 mM KP_i [pH 8.0], 10% [wt/vol] glycerol) to a final total protein concentration of 25 mg/ml. After breaking the cells with a French pressure cell (20,000 lb/in^2), DNA was removed by addition of 0.083% polyethyleneimine that had been equilibrated with buffer A and incubated for 15 min at 4°C. After centrifugation for 15 min at 70,000 × *g*, NaCl and imidazole were added to the supernatant to final concentrations of 400 and 10 mM, respectively. The sample was mixed and incubated with Ni-nitrilotriacetic acid (NTA) resin (~25 mg of protein/ml of resin) for 1 h at 4°C; the resin had been equilibrated with buffer A10 (50 mM KP_i [pH 8], 10% [wt/vol] glycerol, 400 mM NaCl, 10 mM imidazole). Next, the column material was poured into a Bio-Spin column (Bio-Rad Laboratories, Inc.) and washed with 10 column volumes of buffer A10 plus 10 column volumes of buffer A30 (buffer A10 containing 30 mM imidazole at pH 6.0). The protein was eluted with buffer A containing 500 mM imidazole. The fractions eluting from the column were desalted by using PD-10 columns that had been equilibrated with 50 mM Tris-HCl (pH 8.0). Eluted fractions were loaded onto a MonoQ column (HR 5/5; Pharmacia Biotech) that had been equilibrated with 50 mM Tris-HCl (pH 8.0). The proteins were eluted by running a 100-ml gradient of 0 to 500 mM NaCl in 50 mM Tris-HCl (pH 8.0).

Phosphorylation and dephosphorylation assays. For the phosphorylation of IIA$^{LacS-6H}$, 5.8 μM purified IIA$^{LacS-6H}$ was incubated in 50 mM Tris acetate (pH 7.5) containing 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 0.8 mM purified enzyme I, 10 mM PEP, and HPr at concentrations ranging from 1 to 90 μM. The phosphorylation reactions were carried out at 10°C in a total volume of 10 μl. The reactions were stopped by addition of 10 μl of 2× sodium dodecyl sulfate (SDS) sample buffer (29), and the samples were stored on ice. For the dephosphorylation of IIA$^{LacS-6H}$~P, IIA$^{LacS-6H}$ was first phosphorylated by PEP, enzyme I, and HPr, after which these components were removed by binding IIA$^{LacS-6H}$~P to the Ni-NTA resin. Briefly, 70 μl of reaction mixture was mixed with 40 μl of Ni-NTA that had been equilibrated with 50 mM KP_i (pH 7.0). Following a wash with 2 ml of 50 mM KP_i, IIA$^{LacS-6H}$~P was eluted with 120 μl of 50 mM KP_i (pH 7.0)–500 mM imidazole. For the dephosphorylation reactions, IIA$^{LacS-6H}$~P (10 mM or as indicated otherwise) was incubated in 50 mM Tris acetate (pH 7.5)–1 mM DTT–2 mM $MgCl_2$ and HPr in the range of 1 to 100 μM in a total volume of 10 ml at 10°C. The reaction was monitored as described above. The amount of (de)phosphorylated protein was determined by SDS-polyacrylamide gel electrophoresis (PAGE) analysis (15% polyacrylamide [10]) and Coomassie brilliant blue staining (29), and the amounts of IIA$^{LacS-6H}$ and IIA$^{LacS-6H}$~P were determined by densitometry using a Dextra DF2400T scanner (Dextra Technology, Inc.).

Immunological methods. Immunodetection of wild-type and mutant IIA^{LacS} was performed with antibodies raised against a peptide corresponding to the carboxyl-terminal 17 residues of LacS (17) or antibodies raised against purified IIA$^{LacS-6H}$ (this work). Immunodetection of HPr from *S. thermophilus* was performed with antibodies raised against HPr of *S. salivarius*. The proteins were separated by SDS-PAGE (15% polyacrylamide gel) and transferred to polyvinylidene difluoride membranes by semidry electrophoretic blotting. A Western-light chemiluminescence detection kit (Tropix Inc.) was used to visualize the proteins.

Miscellaneous. Uptake of labeled carbohydrates in intact cells was carried out as described by Postma (21). Protein quantification was performed by the D_c protein assay (Bio-Rad), using bovine serum albumin as the standard. N-terminal sequencing of proteins was performed by Eurosequence, Inc., Groningen, The Netherlands.

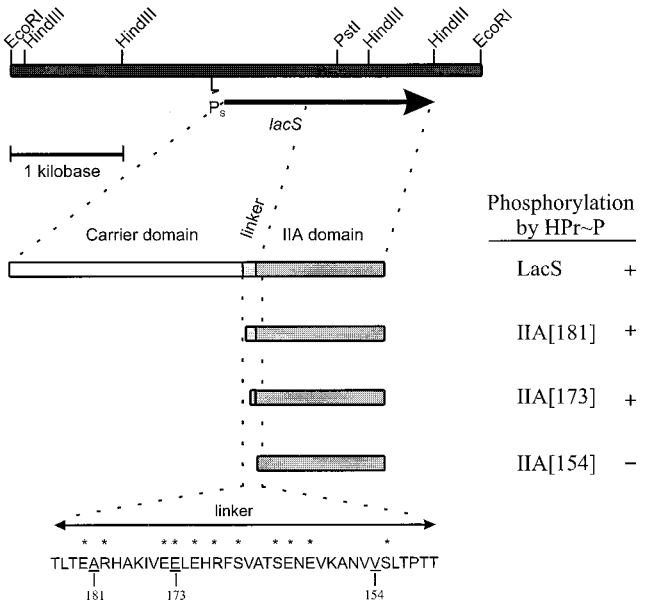

FIG. 2. Construction of gene fragments specifying IIA^{LacS}. The 4.1-kb *Eco*RI chromosomal DNA fragment of pSKE8, containing the *lacS* gene of *S. thermophilus*, is shown. Only relevant restriction sites are indicated. Shown are the *lacS* promoter (P_S), the *lacS* gene (arrow), wild-type protein and portions of the IIA domain (bars). The putative linker and flanking regions are indicated by their amino acid sequences. Amino acids that frequently occur in a Q linker are marked by asterisks, and the residues following the Met of IIA^{LacS}[181], IIA^{LacS}[173], and IIA^{LacS}[154] are indicated by the corresponding numbers. Phosphorylation of the protein was performed in the presence of enzyme I, HPr, and PEP (for details, see Materials and Methods). The ability of LacS to become phosphorylated by HPr~P was taken from reference 19.

Materials. D-[U-^{14}C]glucose (293 mCi/mmol) and [U-^{14}C]glycerol (150 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, United Kingdom. QIA quick spin columns and Ni-NTA resin were purchased from Qiagen, the Bio-Spin columns were from Bio-Rad Laboratories, PD-10 and MonoQ columns (HR 5/5) were from Pharmacia Biotech, 2-deoxy-D-glucose grade II and polyethyleneimine were from Sigma, and the enzymes needed for DNA manipulations were obtained from Boehringer Mannheim. All other materials were reagent grade and obtained from commercial sources.

RESULTS

Expression of the IIA^{LacS}. To study the functional properties of the IIA^{LacS}, this portion of the protein was expressed separately from the carrier domain. Although IIA^{LacS} is homologous to *E. coli* IIA^{Glc} and various other IIA proteins, the similarity at the amino-terminal end is not significant and the start of the linker region, connecting the carrier and IIA domain of LacS, is not well defined (20, 33). Therefore, we selected three translation initiation sites near the linker region such that IIA^{LacS} proteins 181, 173, and 154 amino acids long were obtained (Fig. 2). The individual proteins were tested for the ability to be phosphorylated by HPr~P, which was determined by monitoring the migration of the proteins on an SDS-polyacrylamide gel (4). The IIA^{LacS} proteins present in the cell extracts were detected by immunoblotting using an antibody directed against the carboxyl terminus of LacS. On an SDS-polyacrylamide gel, the IIA^{LacS} proteins migrated at a somewhat higher apparent molecular mass than predicted from the deduced amino acid sequence, which has also been observed for other IIA proteins (27). For IIA^{LacS}[173] and IIA^{LacS}[181], but not for IIA^{LacS}[154], a shift in the migration of the protein upon SDS-PAGE was observed after phosphorylation, indicating that IIA^{LacS}[173] and IIA^{LacS}[181] are phosphorylated by HPr~P. Further experiments were performed with IIA^{LacS}[173], i.e., the smallest fragment that could be phosphorylated and having an amino terminus of the same length as that of IIA^{Glc}.

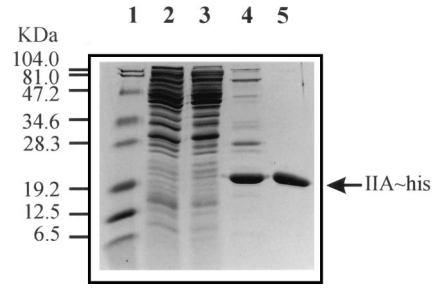

FIG. 3. Purification of IIA$^{LacS-6H}$. Shown is a Coomassie brilliant blue-stained SDS–15% polyacrylamide gel. Lane 1, protein marker; lane 2, cell extract of *E. coli* DH5α/pSKIIAhis (50 μg of protein); lane 3, cytosolic fraction of *E. coli* DH5α/pSKIIAhis (50 μg of protein); lane 4, IIA$^{LacS-6H}$ after nickel chelate affinity chromatography (~10 μg of protein); lane 5, IIA$^{LacS-6H}$ after nickel chelate and anion-exchange chromatography (~7 μg of protein).

To amplify the expression of IIA^{LacS}[173], we made several plasmid constructs (Table 1) in which the copy number was either high or medium and the promoter was inducible (*tac* and T7) or constitutive (*lacS*). After transformation of the *E. coli* hosts (DH5α, MC1061, and BL21) with the appropriate plasmids, the highest expression (2 to 5% of total cell protein) was obtained from the *lacS* promoter present in plasmid pSKIIA with *E. coli* DH5α as the host (data not shown). Immunoblot detection using an antibody directed against the carboxyl terminus of LacS demonstrated that the expressed proteins corresponded to IIA^{LacS} (data not shown). To facilitate the purification of IIA^{LacS}, we constructed pSKIIAhis, in which sequences specifying a factor Xa cleavage site and a six-histidine tag are present at the 3′ end of the IIA^{LacS} gene fragment. The introduction of a carboxyl-terminal His tag had no effect on the ability of HPr~P to phosphorylate IIA^{LacS} (data not shown). On SDS-PAGE, IIA$^{LacS-6H}$ migrated somewhat more slowly than IIA^{LacS}.

Purification of IIA$^{LacS-6H}$. For the purification of IIA$^{LacS-6H}$, *E. coli* DH5α/PSKIIAhis cells were grown to late exponential growth phase (Fig. 3, lane 2). After the cells were broken in a French pressure cell and cell debris/membranes and DNA were removed (Fig. 3, lane 3), the His-tagged protein could be isolated with a purity of 90% by nickel chelate affinity chromatography (lane 4). Further purification was achieved by anion-exchange chromatography using a MonoQ column (lane 5). The protein was eluted at approximately 150 mM NaCl. From 1 liter of cells grown to an A_{660} of 4.5 in a computer-controlled fermentor and under vigorous aeration, 1.6 mg of IIA$^{LacS-6H}$ was obtained. The amino-terminal sequence of the purified protein was Met-Glu-Leu-Glu-His-Arg, which is identical to the anticipated amino acid sequence (20); Glu-462 in LacS was replaced by Met to obtain the initiation codon of the IIA domain. Purification of the His-tagged IIA^{LacS}(H552R) and IIA^{LacS}(I548E/G556D) mutant proteins was performed exactly as for IIA^{LacS}.

Purification of HPr. HPr from *S. thermophilus* was analyzed in column fractions by native PAGE (15% polyacrylamide gel) and immunodetection using antibodies raised against HPr of *S. salivarius*. HPr was isolated from cells of *S. thermophilus* ST11 (Δ*lacS*)/pGKhis that were grown to late exponential phase. After the cells were broken, most of the lysozyme present in the cell lysate was removed by adsorption to S-Sepharose. Subsequently, HPr was purified to near homogeneity in two

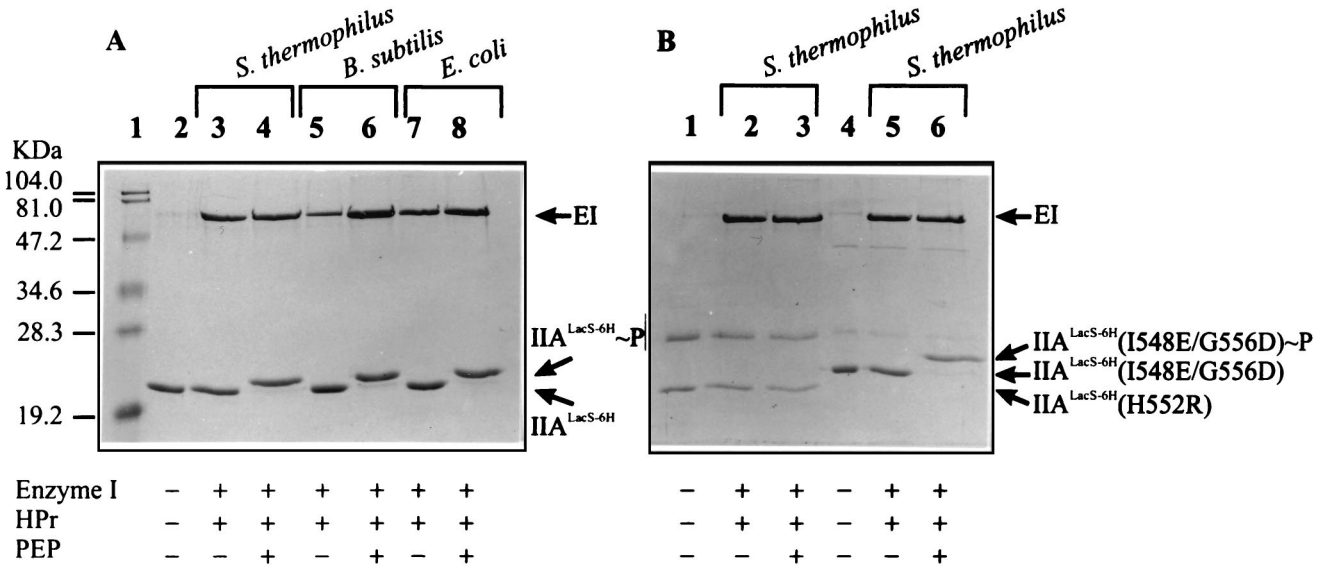

FIG. 4. Phosphorylation of $IIA^{LacS\text{-}6H}$, $IIA^{LacS\text{-}6H}$(H552R), and $IIA^{LacS\text{-}6H}$(I548E/G556D). The Coomassie brilliant blue-stained SDS–15% polyacrylamide gel represents samples containing 0.8 μM enzyme I (EI) from *B. subtilis*, 12.5 μM HPr, 5.8 μM $IIA^{LacS\text{-}6H}$, and/or 10 μM PEP, as indicated at the bottom. The phosphorylation reactions (at 37°C for 15 min) were carried out in 50 mM Tris-acetate (pH 7.5)–1 mM DTT–2 mM $MgCl_2$. (A) IIA^{LacS}. (B) Lanes 1 to 3, IIA^{LacS}(H552R); lanes 4 to 6, $IIA^{LacS\text{-}6H}$(I548E/G556D). The sources of HPr (*S. thermophilus*, *B. subtilis*, and *E. coli*) are indicated above the lanes.

steps, involving anion-exchange and cation-exchange chromatography. On an SDS-polyacrylamide gel, HPr migrated at ~13 kDa. This apparent molecular mass of HPr from *S. thermophilus* is in the same range as found for HPr purified from other streptococci, 6.7 to 17 kDa, while the molecular mass from the nucleotide sequence is 8.9 kDa (35).

Phosphorylation of $IIA^{LacS\text{-}6H}$ by PTS-mediated enzymes. Phosphorylation of $IIA^{LacS\text{-}6H}$ was analyzed by SDS-PAGE. Figure 4A shows the relative electrophoretic mobilities of purified $IIA^{LacS\text{-}6H}$ in the absence (lane 2) and presence of PEP, enzyme I, and HPr of *S. thermophilus* (lane 4), *B. subtilis* (lane 6), and *E. coli* (lane 8). The phosphorylated form of $IIA^{LacS\text{-}6H}$ exhibited a somewhat lower electrophoretic mobility than the nonphosphorylated protein (compare lanes 2 with lanes 4, 6, and 8). These experiments clearly indicate that the IIA domain of LacS can be phosphorylated via PEP and the general PTS energy-coupling proteins enzyme I and HPr of *S. thermophilus* as well as HPr of both *E. coli* and *B. subtilis*. Figure 4B shows the electrophoretic mobilities of the purified mutants $IIA^{LacS\text{-}6H}$(H552R) and $IIA^{LacS\text{-}6H}$(I548E/G556D). The mobility of $IIA^{LacS\text{-}6H}$(H552R) is similar to that of the wild type, whereas $IIA^{LacS\text{-}6H}$(I548E/G556D) exhibited a significantly lower mobility (lane 5). Upon addition of PEP, enzyme I of *B. subtilis*, and HPr of *S. thermophilus*, the migration of $IIA^{LacS\text{-}6H}$(H552R) was not affected (compare lanes 1, 2, and 3), whereas $IIA^{LacS\text{-}6H}$(I548E/G556D) migrated more slowly (compare lanes 5 and 7). Similar results were obtained with PEP, enzyme I, and HPr of *E. coli* and *B. subtilis* (data not shown). These results indicate that $IIA^{LacS\text{-}6H}$(I548E/G556D) but not $IIA^{LacS\text{-}6H}$(H552R) is capable of accepting the phosphoryl group from HPr~P.

Kinetics of phosphorylation and dephosphorylation of $IIA^{LacS\text{-}6H}$ and $IIA^{LacS\text{-}6H}$(I548E/G556D). The phosphorylation kinetics of $IIA^{LacS\text{-}6H}$ by HPr~P from *S. thermophilus* is shown in Fig. 5. To determine the phosphorylation rates more precisely, the experiments were performed at 10°C, which decreased the phosphorylation rate approximately 1 order of magnitude compared to the level at 37°C. In these experiments, the concentration of HPr~P was 5- to 50-fold greater than that of $IIA^{LacS\text{-}6H}$, and the kinetics of $IIA^{LacS\text{-}6H}$ phosphorylation could be approximated as a first-order process (Fig. 5A). The initial rates of phosphorylation of $IIA^{LacS\text{-}6H}$ at different HPr~P concentrations and for HPr of *S. thermophilus*, *E. coli*, and *B. subtilis* are presented in Fig. 5B. Clearly, $IIA^{LacS\text{-}6H}$ was phosphorylated approximately 1 and 2 orders of magnitude faster by HPr~P from *S. thermophilus* than by HPr~P from *E. coli* and *B. subtilis*, respectively. The derived rate constants obtained from these data are summarized in Table 2 (first column).

The $IIA^{LacS\text{-}6H}$(I548E/G556D) mutant was constructed because the Glu and Asp residues are conserved in all PTS members of the IIA family, whereas they are replaced by neutral residues in the non-PTS IIA domains. These residues are predicted to affect the interaction of IIA with its partner molecules, e.g., HPr~P and IIB. The phosphorylation kinetics of $IIA^{LacS\text{-}6H}$(I548E/G556D) is shown in Fig. 5C and Table 2 (fourth column). Indeed, the rate of phosphorylation of $IIA^{LacS\text{-}6H}$(I548E/G556D) by HPr~P from *S. thermophilus* increased approximately 1 order of magnitude. Remarkably, however, the rate of phosphorylation of $IIA^{LacS\text{-}6H}$(I548E/G556D) by HPr~P from *B. subtilis* and *E. coli* increased 2 and 3 orders of magnitude relative to that of $IIA^{LacS\text{-}6H}$, and the phosphorylation became nearly independent of the source of HPr~P.

IIA^{Glc}~P can be dephosphorylated by transferring the phosphoryl group to $IICB^{Glc}$ or by redirecting the phosphoryl group to HPr. The dephosphorylation properties of $IIA^{LacS\text{-}6H}$~P and $IIA^{LacS\text{-}6H}$(I548E/G556D)~P were studied with HPr from *S. thermophilus*, *B. subtilis*, and *E. coli* as phosphoryl acceptors. Figure 6 shows the time course for the phosphoryl transfer from $IIA^{LacS\text{-}6H}$~P to HPr from *S. thermophilus* at different concentrations of HPr. Under these conditions, $IIA^{LacS\text{-}6H}$~P was quite stable and the first-order rate constant for the autodephosphorylation was 6.7×10^{-5} s^{-1} (Fig. 6, inset). The dephosphorylation rates of $IIA^{LacS\text{-}6H}$~P were determined with HPr from *S. thermophilus*, *B. subtilis*, and *E. coli* as phosphoacceptors. As anticipated from the phosphorylation assays, HPr from *S. thermophilus* was a much better acceptor than HPr

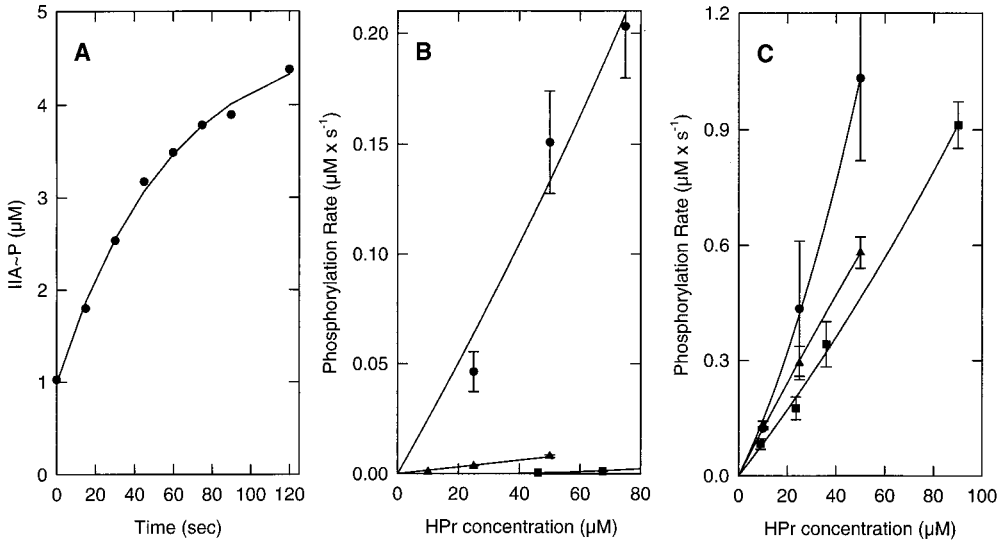

FIG. 5. Phosphoryl transfer from HPr~P of *S. thermophilus*, *B. subtilis*, and *E. coli* to IIA$^{LacS-6H}$ and IIA$^{LacS-6H}$(I548E/G556D). (A) Time course of the phosphoryl transfer reaction between 25 μM HPr~P from *S. thermophilus* and IIA$^{LacS-6H}$. (B) Phosphorylation rate of IIA$^{LacS-6H}$ by HPr~P from *S. thermophilus* (●), *B. subtilis* (■), and *E. coli* (▲). (C) Phosphorylation rate of IIA$^{LacS-6H}$(I548E/G556D) by HPr~P from *S. thermophilus* (●), *B. subtilis* (■), and *E. coli* (▲). The phosphorylation reaction was started by adding 5.8 μM purified IIA$^{LacS-6H}$ or 4.1 μM IIA$^{LacS-6H}$(I548E/G556D) to 50 mM Tris acetate (pH 7.5) containing 1 mM DTT, 2 mM $MgCl_2$, 0.8 μM purified enzyme I, 10 mM PEP, and HPr at concentrations ranging from 0 to 90 μM, in a total volume of 10 μl at 10°C. The reactions were stopped by addition of 10 μl of 2× SDS sample buffer.

from *B. subtilis* or *E. coli* (Table 2). The phosphoryl transfer from IIA$^{LacS-6H}$(I548E/G556D)~P to HPr from *S. thermophilus*, *B. subtilis*, and *E. coli* was too fast to be measured accurately at 10°C. The data with these three proteins as acceptors were qualitatively very similar, showing that residues Glu-548 and Asp-556 allow a better interaction between IIA$^{LacS-6H}$ and HPr of both homologous and heterologous origin.

Complementation of *crr* strains in *trans*. Since *E. coli* HPr~P could phosphorylate IIA^{LacS}, we next addressed the question of whether IIA^{LacS} could substitute for IIA^{Glc} in the phosphoryl transfer catalyzed by the glucose PTS. The phosphoryl transfer activity of IIA^{LacS} was studied in *E. coli* LM1 (*crr manA*), a strain defective in glucose transport due to a lack of IIA^{Glc} as well as a functional mannose PTS. Since the N-terminal residues of IIA^{LacS} could be important for the interaction with the membrane IICB^{Glc} protein, we tested IIA^{LacS}[181] and IIA^{LacS}[173] as well as IIA$^{LacS-6H}$(I548E/G556D) for the ability to restore glucose transport in *E. coli* LM1 (data not shown). The results were all negative, suggesting that these IIA^{LacS} proteins are unable to transfer the phosphoryl group rapidly enough to IICB^{Glc} even though they can be phosphorylated by HPr~P.

To study the ability of IIA^{LacS} to affect glycerol utilization by inhibiting glycerol kinase, IIA^{LacS}[173], IIA$^{LacS-6H}$(H552R), and IIA$^{LacS-6H}$(I548E/G556D) were expressed in *S. typhimurium* PP2178 (*crr*::Tn*10 nagE*). This strain lacks IIA^{Glc} as well as IICBA^{Nag}, and consequently glycerol uptake is not inhibited by the presence of a PTS sugar (Fig. 7A), which was observed in the wild-type strain. Upon transformation of this strain with a plasmid bearing a wild-type *crr*$^+$ or *nagE*$^+$ gene and expressing the corresponding protein, glycerol uptake is inhibited by glucose (or analogs) (36). Under these conditions, the equilibrium of IIA^{Glc} (or II^{Nag}) is shifted from the phos-

TABLE 2. Rate and equilibrium constants for phosphoryl transfer between HPr of *S. thermophilus*, *B. subtilis*, or *E. coli* and IIA^{LacS} or IIA^{LacS}(I548E/G556D)[a]

Source of HPr	IIA^{LacS}			IIA^{LacS} (I548E/G556D), k_1 (M^{-1} s^{-1})
	k_1 (M^{-1} s^{-1})	k_{-1} (M^{-1} s^{-1})	K_{eq} (k_1/k_{-1})	
S. thermophilus	430 ± 70	1,100 ± 600	0.5 ± 0.17	4,000 ± 700
B. subtilis	2.4 ± 0.45	12 ± 6	0.2	3,500 ± 150
E. coli	26 ± 1.6	130 ± 15	0.2	2,900 ± 40

[a] Results are means ± standard errors.

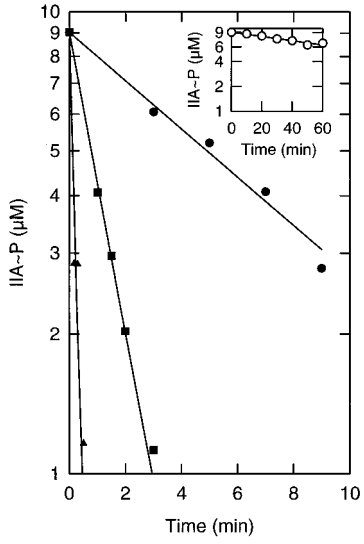

FIG. 6. Phosphoryl transfer from IIA$^{LacS-6H}$~P to HPr from *S. thermophilus*. The dephosphorylation reaction was started by adding 11 μM purified IIA$^{LacS-6H}$~P to 50 mM Tris acetate (pH 7.5) containing 1 mM DTT, 2 mM $MgCl_2$, and HPr at concentrations of 10 μM (●), 25 μM (■), and 50 μM (▲). The reaction volume was 10 μl, and the temperature was 10°C. The inset shows the dephosphorylation of the IIA$^{LacS-6H}$ in the absence of HPr.

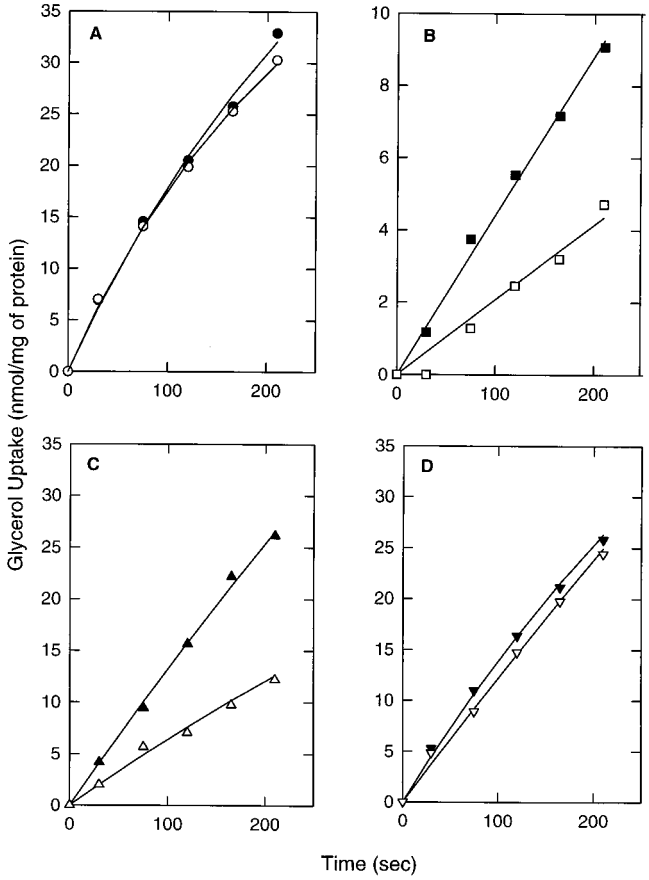

FIG. 7. Inhibition of glycerol kinase activity by IIA^{LacS}. *S. typhimurium* PP2178 (*crr*::Tn*10 nagE*) was transformed with pKK223-3 (control) (A), pKKELE (IIA^{LacS}) (B), pSKIIAm2his [$IIA^{LacS\text{-}6H}$(I548E/G556D)] (C), and pSKIIAm1his [$IIA^{LacS\text{-}6H}$(H552R)] (D). Cells were grown overnight on a minimal salts medium supplemented with 0.4% DL-lactate. After being washed with minimal salts medium, the cells were diluted to an A_{660} of 0.35 in minimal salts medium containing 54 mM glycerol and incubated for 30 min at 37°C; glucose was added to a final concentration of 10 mM; the cells were incubated for 1 h at 37°C and then washed with minimal salts medium. Uptake assays with 0.5 mM [^{14}C]glycerol were performed in the presence (open symbols) and absence (filled symbols) of 10 mM 2-deoxy-D-glucose; the cells were equilibrated in the presence or absence of 2-deoxy-D-glucose for 5 min prior to the initiation of uptake.

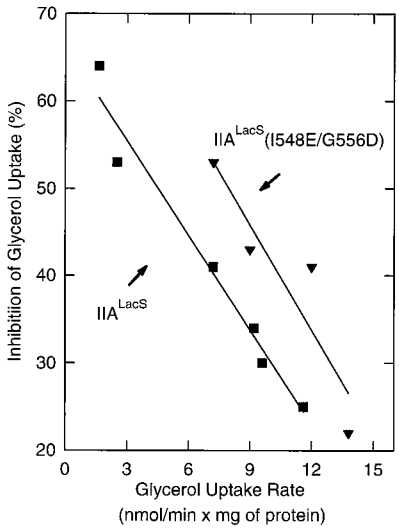

FIG. 8. Relationship between the activity of glycerol kinase and the extent of inhibition of glycerol uptake by IIA^{LacS} (■) and $IIA^{LacS\text{-}6H}$(I548E/G556D) (▼). Cells were grown as described in the legend to Fig. 7. The data are from several independent experiments that reflect partial induction by glycerol for 15, 20, or 30 min. The glycerol kinase activity was measured as [^{14}C]glycerol uptake rate in the absence of 2-deoxyglucose, and the inhibition of [^{14}C]glycerol uptake was measured from the ratio of the rates in the presence and absence of 10 mM 2-deoxyglucose.

phorylated to the dephosphorylated state, which is known to interact with (and inhibit) glycerol kinase (22). Similarly, upon addition of 2-deoxyglucose to *S. typhimurium* PP2178 expressing IIA^{LacS} or $IIA^{LacS\text{-}6H}$(I548E/G556D), glycerol uptake was partially inhibited (Fig. 7B and C). The glycerol uptake was not inhibited by $IIA^{LacS\text{-}6H}$(H552R), indicating that the residue at position 552 in IIA^{LacS} is important for the interaction with glycerol kinase (Fig. 7D). The inhibition of glycerol uptake was most clearly observed when the actual uptake rate of glycerol (amount of glycerol kinase) was low (Fig. 8), indicating that the extent of inhibition is determined by the level of glycerol kinase, as has been observed previously (37). The maximal inhibition of the glycerol uptake rate in *S. typhimurium* PP2178 was 65%. $IIA^{LacS\text{-}6H}$(I548E/G556D) inhibited glycerol kinase to a greater extent than IIA^{LacS} at equal glycerol uptake rates (Fig. 8). This difference is most likely due to the higher expression of $IIA^{LacS\text{-}6H}$(I548E/G556D) than of IIA^{LacS}[173], using the expression plasmids pSKIIAm2his and pKKELE, respectively. IIA^{LacS} could not be expressed to similar high levels in *S. typhimurium* PP2178, as pSKIIAhis was lethal to these cells.

To investigate a possible role of IIA^{LacS} in regulating adenylate cyclase activity, IIA^{LacS}, $IIA^{LacS\text{-}6H}$(H552R), and $IIA^{LacS\text{-}6H}$(I548E/G556D) were expressed in *S. typhimurium* PP2178 and grown on minimal salts A plates containing succinate or citrate. No growth was observed unless 5 mM cAMP was included in the medium. These results indicate that even though these IIA^{LacS} proteins can be phosphorylated by HPr~P, they cannot stimulate adenylate cyclase to restore growth of *crr* mutants on citrate or succinate.

DISCUSSION

In this paper, we describe the functional expression and purification to near homogeneity of the IIA domain of the lactose transport protein of *S. thermophilus*. On the basis of the similarities in the primary sequences of IIA^{LacS}, IIA^{Glc}, and other PTS IIA protein domains (12, 20), we anticipated that IIA^{LacS} would be able to carry out one or more functions associated with IIA^{Glc}, i.e., complement *crr* strains in PTS-mediated glucose uptake, inhibit glycerol kinase, and/or activate adenylate cyclase.

The in vitro phosphorylation assays indicate that $IIA^{LacS\text{-}6H}$ can be phosphorylated by HPr~P from *S. thermophilus*, *B. subtilis*, and *E. coli*, and the results suggest that the phosphorylation site in IIA^{LacS} is His-552. This residue corresponds to His-90 of IIA^{Glc} in *E. coli*, which has been shown to be the phosphoryl-accepting site (4). Although IIA^{LacS} can be phosphorylated by HPr~P, the phosphorylation rates are lower than for phosphorylation of IIA proteins involved in PTS-mediated transport (14). The rate constant for the phosphoryl transfer between *S. thermophilus* HPr~P and IIA^{LacS} at a temperature of 10°C was $k_1 = 4.3 \times 10^2\ M^{-1}\ s^{-1}$; the rate constant for the reverse reaction (IIA^{LacS}~P to HPr) was $k_{-1} = 1.1 \times 10^3\ M^{-1}\ s^{-1}$. Although these phosphoryl transfer rates increased 10-fold at 37°C, it is evident that phosphorylation of IIA^{LacS} by HPr~P from *S. thermophilus* is 4 orders of magnitude slower than phosphorylation of IIA^{Glc} by HPr~P from *E.*

coli (14) ($k_1 = 6.1 \times 10^7\ M^{-1}\ s^{-1}$). With HPr~P from *B. subtilis* and *E. coli*, the differences are even much larger, as one might expect, when interacting proteins from different sources are compared (Fig. 5). The phosphorylation rate of IIA^{LacS} by HPr~P from *S. thermophilus* increased 1 order of magnitude when Ile-548 and Gly-556 were substituted by Glu and Asp, respectively, and increased 3 and 4 orders of magnitude when $IIA^{LacS\text{-}6H}$(I548E/G556D) was phosphorylated by HPr~P from *E. coli* and *B. subtilis*, respectively (Fig. 6). The corresponding acidic residues (Glu-86 and Asp-94 of IIA^{Glc} in *E. coli*) are conserved in all PTS IIA protein domains but are not present in the non-PTS IIA protein domains (Fig. 1). The crystal structure of IIA^{Glc} from *E. coli* shows that Asp-94 participates in phosphoryl transfer, as the backbone amide nitrogen of this residue is positioned to form an H bond to the phosphoryl group (16). It has been suggested that this H bond stabilizes the transition state (trigonal bipyramid) form of the P atom. The conserved Glu-86 is more exposed to the surface of the molecule and is proposed to serve as the recognition site for one of the interacting PTS proteins (12). Substitution in the β-glucoside IICBA of *E. coli* of Asp-551, the equivalent of Asp-94 in IIA^{Glc}, by an alanine residue decreased the phosphorylation rate relative to the wild-type protein (30). The higher rate of phosphorylation of $IIA^{LacS\text{-}6H}$(I548E/G556D) than of $IIA^{LacS\text{-}6H}$ is consistent with the predictions one could make from the three-dimensional structure of IIA^{Glc} of *E. coli* and substantiates the critical role of these residues in the phosphorylation of IIA. Importantly, the rates of (de)phosphorylation of $IIA^{LacS\text{-}6H}$(I548E/G556D) by HPr~P from *E. coli* and *B. subtilis* are similar to those of *S. thermophilus*, which allowed us to assess some of the functional properties of IIA^{LacS} in a heterologous system (see below).

The inability of IIA^{LacS} to substitute for IIA^{Glc} in phosphorylation of *E. coli* IIB, as determined by assays of PTS-mediated glucose uptake, is likely due to the low phosphorylation rate of IIA^{LacS} by *E. coli* HPr~P. Additionally, the inability of IIA^{LacS} to complement the lack of IIA^{Glc} in PTS-mediated glucose uptake could also be due to an incompatible amino terminus. In this respect, it is worth noting that cell extracts of *E. coli* contain two electrophoretically distinguishable forms of IIA^{Glc}, a slow form and fast form. The fast form [IIA^{Glc}(fast)] is the product of an endopeptidase that cleaves the N-terminal heptapeptide from the mature form. IIA^{Glc}(fast) is fully active in accepting the phosphoryl group from HPr~P, but it has only 3% of the phosphodonor activity of the intact protein (13) and has a smaller effect on inhibition of methyl-β-D-thiogalactopyranoside uptake (inducer exclusion) than IIA^{Glc}(slow) (15). This finding suggests that the N-terminal region of IIA^{Glc} could participate in the interaction with its partner molecules, such as IIB^{Glc} and the lactose permease. The IIA^{LacS} protein that was used in the majority of the experiments is 173 amino acids long and corresponds in length to IIA^{Glc}(slow). Addition of another eight amino acids (part of putative Q-linker region in LacS) did not affect the (de)phosphorylation activity. Shortening of IIA^{LacS}[173] by 19 residues abolished the ability of the protein to accept the phosphoryl group from HPr. Future experiments are required to clarify which amino acid residues are involved in the interaction between IIA^{Glc} and IIB^{Glc} to facilitate phosphoryl transfer to glucose.

Apart from its function in the uptake and phosphorylation of sugars, IIA^{Glc} interacts with several non-PTS enzymes such as glycerol kinase, the MalK component of the maltose transport system, and the melibiose and lactose transporters of *E. coli*, resulting in inactive complexes (23). In a previous study (36), it was shown that wild-type LacS protein, in which the IIA domain is fused to the membrane-bound carrier domain, does not inhibit glycerol kinase. This could point to the inability of membrane-bound IIA^{LacS} to interact functionally with glycerol kinase, but more likely the expression of LacS in *S. typhimurium* was too low for significant inhibition to be observed. It is worth noting that membrane-bound IIA^{Nag}, as part of the $IICBA^{Nag}$ complex, is able to inhibit glycerol kinase (37), indicating that inducer exclusion is not exclusively mediated by cytosolic IIA^{Glc}. Since we could express IIA^{LacS} separate from the carrier domain of LacS, we also studied the ability of the various IIA^{LacS} proteins to inhibit glycerol uptake. Although IIA^{LacS}~P and $IIA^{LacS\text{-}6H}$ (I548E/G556D)~P were not able to rapidly transfer the phosphoryl group to $IICB^{Glc}$, the uptake of glycerol in *S. typhimurium* PP2178 was inhibited by the addition of 2-deoxyglucose, most likely because IIA^{LacS}~P or $IIA^{LacS\text{-}6H}$ (I548E/G556D)~P was dephosphorylated through the redirection of phosphoryl groups to HPr and subsequently to the mannose PTS. 2-Deoxyglucose is taken up by the mannose PTS and thus indirectly influences the phosphorylation state of IIA^{LacS}. The extent of inhibition of glycerol uptake was dependent on the actual uptake rate of glycerol (amount of glycerol kinase) (Fig. 8), suggesting that IIA^{LacS} inhibits glycerol kinase by forming a stoichiometric complex with the enzyme as observed for IIA^{Glc} and glycerol kinase (37). Hurley et al. (7) identified the sites of interactions between glycerol kinase and *E. coli* IIA^{Glc}, which mainly involve hydrophobic and electrostatic interactions and a Zn(II) binding site. The Zn(II) binding site is made up of the two active-site histidines of IIA^{Glc} (His-75 and His-90), Glu-478 of glycerol kinase, and an H_2O molecule. In the absence of Zn(II), IIA^{Glc} binds to glycerol kinase primarily via the hydrophobic patch and without participation of the His residues. Except for the histidines that coordinate the Zn atom, the positions are poorly conserved in the non-PTS IIA protein domain(s). Nevertheless, IIA^{LacS} was able to inhibit glycerol kinase. Our results also indicate that substitution of His-552 for Arg (His-552 in LacS corresponds to His-90 in *E. coli* IIA^{Glc}) abolished the putative interaction of IIA^{LacS} with glycerol kinase. Possibly, the binding constant of the mutant to glycerol kinase is decreased as suggested for IIA^{Glc}(H90Q) (16). Whether the Zn atom plays an important role in the interaction of IIA proteins to glycerol kinase is unclear, as IIA^{Glc}(H75Q) seems as effective an inhibitor as the wild-type protein (cited in reference 16).

In gram-negative enteric bacteria the phosphorylated form of IIA^{Glc} stimulates adenylate cyclase, whereby the expression of many catabolic enzymes is regulated through changes in cAMP levels, e.g., the expression of genes for succinate and citrate catabolism (1, 23). Reddy et al. (25) suggested that the acquisition of a negative charge at His-90~P of *E. coli* IIA^{Glc} is responsible for the interaction with adenylate cyclase. Although IIA^{LacS} and IIA^{LacS}(I548E/G556D) could be phosphorylated by HPr from *E. coli* both in vitro and in vivo, these IIA^{LacS} proteins were not able to stimulate adenylate cyclase sufficiently, if at all, to restore growth of *crr* mutants on succinate and citrate. The in vivo phosphorylation is suggested by the identification of two species of IIA^{LacS} (IIA^{LacS} and IIA^{LacS}~P) in cell extracts of *E. coli* cells (unpublished data); it also follows from the glycerol kinase inhibition experiments.

The equilibrium constant ($K_{eq} = k_1/K_{-1}$) for the phosphoryl transfer reactions between IIA^{LacS} and HPr from *S. thermophilus* is 0.5, which is approximately threefold lower than the corresponding value for HPr and IIA^{Glc} from *E. coli* ($K_{eq} = 1.4 \pm 0.5$ [14]). This implies that at a given phosphorylation potential, the phosphorylation state of IIA^{LacS} (and we assume LacS as well) in *S. thermophilus* will be lower than that of IIA^{Glc} in *E. coli*. Since phosphorylation of LacS inhibited the lactose-H^+ symport reaction (17), this condition will be met in

vivo only when the [HPr(His~P)]/[HPr] ratio is relatively high. Vadeboncoeur and coworkers (34) reported that in stationary-phase cells of *S. salivarius*, the [HPr(His~P)]/[HPr] ratio is approximately 2.5 for *S. salivarius*. On the other hand, actively growing cells of *S. mutans* and *S. salivarius* contained mainly HPr(Ser-P) and HPr(His~P)(Ser-P), with very little HPr(His~P) and free HPr. Thus, the degree of phosphorylation of LacS will be low in exponentially growing cells of *S. thermophilus*, which implies that the system will have full activity. In resting cells of *S. thermophilus*, a partial inhibition of LacS-mediated methyl-β-D-thiogalactopyranoside transport was observed (17).

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