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Phosphorylation of *Streptococcus salivarius* Lactose Permease (LacS) by HPr(His~P) and HPr(Ser-P)(His~P) and Effects on Growth

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The oral bacterium *Streptococcus salivarius* takes up lactose via a transporter called LacS that shares 95% identity with the LacS from *Streptococcus thermophilus*, a phylogenetically closely related organism. *S. thermophilus* releases galactose into the medium during growth on lactose. Expulsion of galactose is mediated via LacS and stimulated by phosphorylation of the transporter by HPr(His~P), a phosphocarrier of the phosphoenolpyruvate:sugar phosphotransferase transport system (PTS). Unlike *S. thermophilus*, *S. salivarius* grew on lactose without expelling galactose and took up galactose and lactose concomitantly when it is grown in a medium containing both sugars. Analysis of the C-terminal end of *S. salivarius* LacS revealed a IIA-like domain (IIA^{LacS}) almost identical to the IIA domain of *S. thermophilus* LacS. Experiments performed with purified proteins showed that *S. salivarius* IIA^{LacS} was reversibly phosphorylated on a histidine residue at position 552 not only by HPr(His~P) but also by HPr(Ser-P)(His~P), a doubly phosphorylated form of HPr present in large amounts in rapidly growing *S. salivarius* cells. Two other major *S. salivarius* PTS proteins, $IIAB_L^{Man}$ and $IIAB_H^{Man}$, were unable to phosphorylate IIA^{LacS}. The effect of LacS phosphorylation on growth was studied with strain G71, an *S. salivarius* enzyme I-negative mutant that cannot synthesize HPr(His~P) or HPr(Ser-P)(His~P). These results indicated that (i) the wild-type and mutant strains had identical generation times on lactose, (ii) neither strain expelled galactose during growth on lactose, (iii) both strains metabolized lactose and galactose concomitantly when grown in a medium containing both sugars, and (iv) the growth of the mutant was slightly reduced on galactose.

Streptococcus salivarius is the predominant bacterial species among the pioneer microorganisms that colonize the mouth (19). Acquisition of and competition for nutrients, particularly sugars, which serve as the major energy source for oral streptococci, constitute vital ecological determinants for the survival of oral bacteria. *S. salivarius* is able to metabolize a broad variety of sugars that can be grouped into two categories, non-PTS sugars, which are taken up by transport systems energized by proton motive force or ATP, and PTS sugars, which are transported by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) (4, 35, 38). The PTS uses phosphoenolpyruvate (PEP) in a group translocation process to phosphorylate incoming mono- and disaccharides via a phosphoryl-transfer cascade involving the non-sugar-specific proteins, Enzyme I (EI) and HPr, and a family of sugar-specific enzyme II complexes (EII) (27). In gram-positive bacteria, the PTS controls sugar metabolism by regulating transporter activities and gene transcription via the protein HPr (6, 29). This protein can be phosphorylated by EI at the expense of PEP on a histidine at position 15, generating HPr(His~P), and by a ATP-dependent protein kinase/phosphorylase, called HPrK/P, on a serine at position 46, generating HPr(Ser-P) (6, 8, 29). Both HPr(His~P) and HPr(Ser-P) possess regulatory functions. HPr(His~P) accomplishes its regulatory functions by reversibly phosphorylating its targets, and HPr(Ser-P) accomplishes its regulatory functions by protein-protein interactions (7, 13, 31, 42). In addition to the aforementioned phosphorylated forms of HPr, rapidly growing streptococcal cells contain substantial amounts of the doubly phosphorylated form HPr(Ser-P)(His~P), whose functions remain unclear (33, 36, 37).

Lactose (milk sugar) is a disaccharide composed of glucose and galactose and is an important energy source for oral streptococci. It is taken up by *S. salivarius* via a non-PTS transport system (14) composed of a single membrane protein, lactose permease (LacS), that possesses an amino acid sequence that shares 95% identity with the sequence of *Streptococcus thermophilus* LacS (40). *S. thermophilus* and *S. salivarius* are closely related and belong, together with *Streptococcus vestibularis*, to the same phylogenetic cluster, forming the *salivarius* group of oral streptococci (16). Most *S. thermophilus* strains are unable to grow on galactose and release galactose into the medium during growth on lactose (15). The release of galactose has even been observed with Gal^+ mutant strains (34). Expulsion of galactose by *S. thermophilus* is mediated via LacS during lactose-galactose exchange, a process that is strengthened after phosphorylation of the transporter at a histidine residue that is part of a IIA domain at the C-terminal end of the protein (11, 17, 23, 24, 41). The phosphorylation, which chiefly occurs at the end of the logarithmic growth phase, is catalyzed by HPr (His~P), whose intracellular concentrations increase at the end of the exponential growth phase (11, 12).

Unlike *S. thermophilus*, *S. salivarius* readily metabolizes ga-

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genotype and/or characteristic(s)	Source or reference
Strains		
S. salivarius		
ATCC 25975	Wild-type, Lac$^+$ Glu$^+$ Gal$^+$	I. R. Hamilton, University of Manitoba
G71	El-negative mutant derived from *S. salivarius* ATCC 25975	9
E. coli		
BL21 (DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (*DE3*)	Novagen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10* (Tet^r)]	Stratagene
LMG194	F$^-$ Δ*lacX74 galE thi rpsL* Δ*phoA* (*Pvu*II) Δ*ara714 leu*::Tn*10*	Invitrogen
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ*80lacZ*Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU rpsL endA1 nupG*	Invitrogen
Plasmids		
pET-28a(+)	Expression vector	Novagen
pET-29a(+)	Expression vector	Novagen
pET-19b	Expression vector	Novagen
pBAD/His	Expression vector	Invitrogen
pCR-Blunt	Cloning vector	Invitrogen
pETI-16	Contains the *ptsI* gene of *S. salivarius* cloned into pET-28a(+)	This work
pLacSIIA	Contains the last 519 nucleotides of *lacS* from *S. salivarius* (which code for the IIA domain), cloned into pET29a(+)	This work
pLacSIIAH552R	Contains the last 519 nucleotides of *lacS* from *S. salivarius* (which code for the IIA domain), with a mutation replacing LacS H552 by R, cloned into pET29a(+)	This work
pTML2	Contains the *manL* gene of *S. salivarius* cloned into pET19b	This work
pDR3	Contains the *manH* gene of *S. salivarius* cloned into pET19b	This work
pHPW18	Contains the *ptsH* gene of *S. salivarius* cloned into pBAD	8
pHPK229	Contains the *hprK* gene of *S. salivarius* cloned into pCR-Blunt	3

lactose and lactose, and growth on lactose is not accompanied by an extracellular accumulation of galactose (40). Moreover, *S. salivarius* cells growing on lactose contain large amounts of HPr(Ser-P)(His~P) during the exponential growth phase (22). The purpose of the present study was to determine whether *S. salivarius* LacS is controlled by phosphorylation and whether the doubly phosphorylated form of HPr, in addition to HPr(His~P), can serve as a phosphate donor.

MATERIALS AND METHODS

Strains and growth conditions. The strains and plasmids used in the present study are listed in Table 1. *S. salivarius* was grown at 37°C as described previously (3). *Escherichia coli* strains were grown with aeration at 37°C in Luria-Bertani medium. When necessary, 20 μg of tetracycline/ml, 50 μg of ampicillin/ml, and/or 30 μg of kanamycin/ml was added. Generation times were determined as described previously (22). To determine sugar utilization by growing cells, cells were grown in tubes containing 10 ml of medium, and 0.2-ml samples were removed at intervals, heated at 100°C for 10 min to stop metabolism, centrifuged to remove cells, and stored at −20°C until sugar assays were conducted.

DNA purification and manipulations. Chromosomal DNA was isolated from streptococci as described by Gauthier et al. (9). Unless otherwise mentioned, DNA manipulations were performed by standard procedures (1). *E. coli* BL21(DE3) cells were made competent and transformed with plasmid DNA by electroporation (30). DNA fragments used for sequencing and subcloning were recovered from agarose gels by using a QIAquick purification kit (Qiagen). Unless otherwise specified, the PCRs were performed by using a DNA Thermal Cycler 480 (Perkin-Elmer) in a total volume of 100 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 1 μg of DNA, 0.2 μM concentrations of primers, and 100 μM concentrations (each) of the four deoxynucleotide triphosphates. The reactions were carried out for 30 cycles in the presence of 1 U of *Vent* DNA polymerase (Promega) with the following temperature-time profile: 94°C for 1 min, 54°C for 1 min, and 72°C for 40 s.

Gene cloning. *S. salivarius ptsI*, the gene coding for EI of the PTS, was PCR amplified by using the forward primer ptsI69-N and the reverse primer ptsI1804R-X (Table 2). The amplicon was cloned into the overexpression plasmid pET-28a(+), adding a His_6 tag and cleaving a thrombin site at the N terminus of EI to give plasmid pETI-16. The portion of *S. salivarius lacS* coding for IIA^{LacS} was PCR amplified by using the forward primer IIA173 and the reverse primer IIA173R. Primer IIA173 covered positions 1,373 to 1,405 relative to the adenine of the ATG initiation codon of *S. salivarius lacS* and primer IIA173R covered positions 2,951 to 2,979, including the first eight nucleotides of *lacZ* (40). The amplified DNA fragment comprised a region of *lacS* encoding the entire IIA domain of LacS, as well as 37 amino acids upstream from the IIA domain. The amplicon was cloned into the overexpression plasmid pET-29a(+) (Novagen), adding two amino acids (LE) and a His_6 tag at the C terminus of IIA^{LacS} to give plasmid pLacSIIA. Replacement of IIA^{LacS} His_{552} by Arg was carried out by PCR with pLacSIIA as a template and the QuickChange site-directed mutagenesis kit (Stratagene). The PCR mixture contained 10 ng of pLacSIIA, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 125 ng of the oligonucleotide primers IIA-H91R-F and IIA-H91R-R, and 2.5 U of *Pfu* Turbo DNA polymerase (Promega). After a 30-s incubation at 95°C, the amplification reaction was carried out for 16 cycles, each with a 30-s denaturing step at 95°C, a 1-min annealing step at 54°C, and a 12-min extension step at 68°C. After digestion with *Dpn*I and transformation of *E. coli* XL1-Blue with the resulting mixture, we obtained plasmid pLacSIIAH552R, which bore the same DNA

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]
ptsI69-N	CCGTAAGCATATGACAGAAATGCTTAAA
pts1804R-X	GGCCTCGAGTTTTTAAATGATTAGTCCCTAC
IIA173	TGAAGTACATATGGAATTGGAACATCGCTTTA
IIA173R	GTTCATCTCGAGTTCTCCTTTTTTGAAG
IIA-H91R-F	TTGTTCTTATCC**GAG**TTGGTATCGGAACAGTTAA
IIA-H91R-R	TTAACTGTTCCGATACCAA**CTC**GGATAAGAACAA
manL44	GGAGAACACATATGGGTATCGGTATTAT
manL1041R	ACTAATGGATCCGAATGAAGGTTATTGA
manH53F	GGAAGAACACATATGGGTATCGGTATTAT
manH1071R	AACTCATTTATGCATCCTCGAGAATTA

[a] Underlining indicates nucleotides participating in restriction sites. The nucleotides in boldface indicate the codon replacing His by Arg in IIA^{LacS}.

fragment as pLacSIIA, with a two-nucleotide substitution that replaced His_{552} with Arg. *S. salivarius manL*, which codes for $IIAB_L^{Man}$, was PCR amplified with the forward primer manL44 and the reverse primer manL1041R. *S. salivarius manH*, which codes for $IIAB_H^{Man}$, was PCR amplified with the forward primer manH53F and the reverse primer manH1071R. The amplicons were cloned into the overexpression plasmid pET-19b (Novagen), adding an enterokinase site and a His_{10} tag at the N termini of $IIAB_L^{Man}$ and $IIAB_H^{Man}$, to yield plasmids pTML2 and pDR3, respectively.

Protein purification. EI and HPr were purified from *S. salivarius* as described previously (39). *S. salivarius* HPrK/P, the enzyme that phosphorylates HPr on Ser_{46} at the expense of ATP, was purified without a His tag from *E. coli* bearing pHPK229 as previously reported (3). His_6-HPr was purified from *E. coli* LMG194 bearing pHPW18 (8). EI-, IIA^{LacS}-, $IIA^{LacSH552R}$-, $IIAB_L^{Man}$-, and $IIAB_H^{Man}$-overproducing strains were generated by transforming *E. coli* BL21(DE3) with the plasmids pETI-16, pLacSIIA, pLacSIIAH552R, pTML2, and pDR3, respectively. Cells were grown in 500 ml of medium to the late exponential phase, and expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) according to the manufacturer's instructions (Novagen). Preparation of the cell extracts and purification of the recombinant proteins on an Ni-nitrilotriacetic acid (NTA) Superflow column were performed as described previously (8). His_6-EI was further purified on a Superdex 200 HR column (Pharmacia), *S. salivarius* IIA^{LacS} and $IIA^{LacSH552R}$ were further purified by chromatography on a MonoQ HR 5/5 column (Pharmacia), and His_{10}-$IIAB_L^{Man}$ and His_{10}-$IIAB_H^{Man}$ were further purified by chromatography on a Superdex 75HR column (Pharmacia). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses showed that the EI and the IIA^{LacS} proteins were >99% pure, whereas His_{10}-$IIAB_L^{Man}$ and His_{10}-$IIAB_H^{Man}$ were >97% pure.

Synthesis of His_6-HPr(Ser-P). The synthesis of His_6-HPr(Ser-P) was carried out by using purified *S. salivarius* HPrK/P (5 μg) and His_6-HPr (500 μg), which were incubated for 60 min at 37°C in 50 mM Tris-HCl (pH 7.5) containing 2 mM ATP and 5 mM $MgCl_2$. The reaction product [His_6-HPr(Ser-P)] was purified on an Ni-NTA column and by size exclusion chromatography on a Superdex 75HR 10/30 column (Pharmacia) equilibrated with 10 mM potassium phosphate (pH 7.5) containing 100 mM NaCl. The purity of the His_6-HPr(Ser-P) was verified by PAGE under native conditions (28).

Phosphorylation of His_6-IIA^{LacS} by His_6-HPr(His~^{32}P) and His_6-HPr(Ser-P)(His~^{32}P). [^{32}P]PEP was prepared according to the method of Mattoo and Waygood (18) by using purified PEP carboxykinase from *E. coli* K-12 HFr 3000, which was kindly provided by A. H. Goldie (University of Saskatchewan). Phosphorylation of His_6-IIA^{LacS} by His_6-HPr(His~P) was carried out in 50 mM Tris-acetate (pH 7.5) containing 1 mM dithiothreitol (DTT), 1 mM $MgCl_2$, 0.8 μM His_6-EI, 24 μM His_6-HPr, 5.8 μM His_6-IIA^{LacS} or His_6-$IIA^{LacSH552R}$, and 1 mM [^{32}P]PEP (30 μCi/μmol). The mixture was incubated at 10°C for 2 min. Samples were withdrawn at intervals, and the reaction was stopped by adding an equal volume of a solution containing 180 mM Tris-HCl (pH 6.8), 200 mM SDS, 30% glycerol, 2 M β-mercaptoethanol, and 0.003% bromophenol blue (stop solution). The proteins were separated by SDS-PAGE and revealed by autoradiography as described previously (28). His_6-HPr(Ser-P)(His~^{32}P) was synthesized in 50 mM Tris-acetate (pH 7.5) containing 4 mM DTT, 4 mM $MgCl_2$, 1.5 μM His_6-EI, and 40 μM His_6-HPr(Ser-P). After the mixture was incubated at 37°C for 10 min, 1 mM [^{32}P]PEP (30 μCi/μmol) was added, and the solution was incubated at 37°C for an additional 45 min. Analysis by SDS-PAGE revealed that 50% of the His_6-HPr(Ser-P) was transformed into His_6-HPr(Ser-P)(His~P) under these conditions. The solution was then incubated at 10°C and His_6-IIA^{LacS} or His_6-$IIA^{LacSH552R}$ was added to a final concentration of 5.8 μM. The reaction products were analyzed as described for the phosphorylation of His_6-IIA^{LacS} by His_6-HPr(His~P).

Phosphorylation of His_6-IIA^{LacS} by His_{10}-$IIAB_L^{Man}$(His~^{32}P) and His_{10}-$IIAB_H^{Man}$(His~^{32}P). $IIAB_L^{Man}$ and $IIAB_H^{Man}$ are PTS proteins phosphorylated on His residues by HPr(His~P) (20). His_{10}-$IIAB_L^{Man}$(His~^{32}P) and His_{10}-$IIAB_H^{Man}$(His~^{32}P) were synthesized by using 10 mM HEPES (pH 7.5) containing 5 mM $MgCl_2$, 0.6 μM EI, 16 μM HPr, 9 μM His_{10}-$IIAB_L^{Man}$ or His_{10}-$IIAB_H^{Man}$, and 1 mM [^{32}P]PEP (180 μCi/μmol). The mixture was incubated at room temperature for 10 min, after which His_{10}-$IIAB_L^{Man}$(His~^{32}P) and His_{10}-$IIAB_H^{Man}$(His~^{32}P) were isolated by chromatography on a 200-μl Ni-NTA Superflow column as described above. His_6-IIA^{LacS} was phosphorylated by His_{10}-$IIAB_L^{Man}$(His~^{32}P) and His_{10}-$IIAB_H^{Man}$(His~^{32}P) in 10 mM HEPES (pH 7.5) containing 5 mM $MgCl_2$, 2.8 μM His_{10}-$IIAB_L^{Man}$(His~^{32}P) or His_{10}-$IIAB_H^{Man}$(His~^{32}P), and 5.8 μM His_6-IIA^{LacS} in a total volume of 30 μl. The mixture was incubated at room temperature for 10 min, and the reaction was stopped by adding 15 μl of the stop solution described above. The proteins were separated by SDS-PAGE and revealed by autoradiography (28).

Dephosphorylation of His_6-IIA^{LacS}(His~^{32}P) by His_6-HPr and His_6-HPr(Ser-P). His_6-IIA^{LacS}(His~^{32}P) was synthesized by using 50 mM Tris-acetate (pH 7.5) containing 1 mM DTT, 2 mM $MgCl_2$, 1.7 μM EI, and 18 μM HPr in a total volume of 270 μl. After a 10-min incubation at 37°C, [^{32}P]PEP was added to the mixture at a final concentration of 1 mM (30 μCi/μmol), and the solution was incubated at 37°C for an additional 25 min. His_6-IIA^{LacS} was then added to the solution to a final concentration of 15 μM, and the incubation was extended for another 25 min to allow the synthesis of His_6-IIA^{LacS}(His~^{32}P). Phosphorylated His_6-IIA^{LacS} was purified on a 260-μl Ni-NTA column equilibrated with 50 mM potassium phosphate (pH 7.0). The column was first washed with 1.4 ml of 50 mM potassium phosphate (pH 7.0), and the His_6-IIA^{LacS}(His~^{32}P) was eluted with the same buffer containing 300 mM imidazole. Analysis by SDS-PAGE revealed that the preparation was devoid of EI and HPr. The dephosphorylation of His_6-IIA^{LacS}(His~^{32}P) by HPr and HPr(Ser-P) was carried out in 50 mM Tris-acetate (pH 7.5) containing 1 mM DTT, 2 mM $MgCl_2$, and either 20 μM HPr or HPr(Ser-P) in a total volume of 15 μl. After the mixture was incubated for 10 min at 37°C, His_6-IIA^{LacS}(His~^{32}P) was added to a final concentration of 2 μM, and the incubation was extended for 5 min. The reaction was stopped by the addition of an equal volume of the stop solution described above. The proteins were separated by SDS-PAGE and revealed as described above.

Sugar and protein assays. Glucose concentrations were measured by using a peroxidase-glucose oxidase assay (Sigma). Galactose was determined by using a peroxidase-galactose oxidase assay (2). Lactose was assayed by measuring the concentration of glucose or galactose in samples both before and after hydrolysis with β-galactosidase for 1 h at 37°C in 233 mM citrate buffer (pH 6.6) containing 60 mM $MgSO_4$ and 0.05 U of β-galactosidase (Worthington)/μl. Protein concentrations were measured by using the method of Peterson (21) with bovine serum albumin as the standard.

RESULTS

Growth of *S. salivarius* on lactose and galactose. The growth of *S. salivarius* in M17 medium supplemented with 4.4 mM lactose does not result in the accumulation of galactose in the medium (40). To determine whether the type of culture medium and the extracellular lactose concentration influences the release of galactose by *S. salivarius*, we looked for an accumulation of galactose in the medium during growth in M17 (Fig. 1A) and TYE (not shown) media containing 50 to 60 mM lactose. *S. salivarius* did not release galactose during growth in either M17 or TYE. When *S. salivarius* was grown in a medium containing a mixture of lactose and galactose, the two sugars were consumed concomitantly and at the same rate (Fig. 1B). When galactose was provided to cells growing on lactose, the galactose was immediately taken up and did not interfere with lactose uptake (Fig. 1C). Thus, unlike Gal^- and Gal^+ strains of *S. thermophilus* (15, 34), *S. salivarius* readily metabolized galactose, even in the presence of lactose.

Amino acid sequence comparison of *S. salivarius* and *S. thermophilus* IIA^{LacS}. The expulsion of galactose by *S. thermophilus* is mediated by LacS and is stimulated when the IIA domain (IIA^{LacS}), which is located at the C-terminal end of the protein, is phosphorylated (11, 23–24). We recently cloned and sequenced the gene coding for *S. salivarius* LacS (40). A comparison of the translated amino acid sequence of the C-terminal of *S. salivarius* LacS with the same sequence from various strains of *S. thermophilus* (LMG18311, A147, and SMQ-301) revealed that *S. salivarius* LacS possessed a C-terminal IIA-like domain that shared >97% identity with *S. thermophilus* orthologues. *S. salivarius* IIA^{LacS} possessed the His residue (His_{552}) that is phosphorylated by HPr(His~P) in *S. thermophilus* (23, 24). However, the amino acid sequence of *S. salivarius* IIA^{LacS} differed from the sequence of *S. thermophilus* IIA^{LacS} at three positions: Ile_{532} was replaced by Val, Asn_{561} was replaced by Lys, and Lys_{616} was replaced by Glu. To determine whether

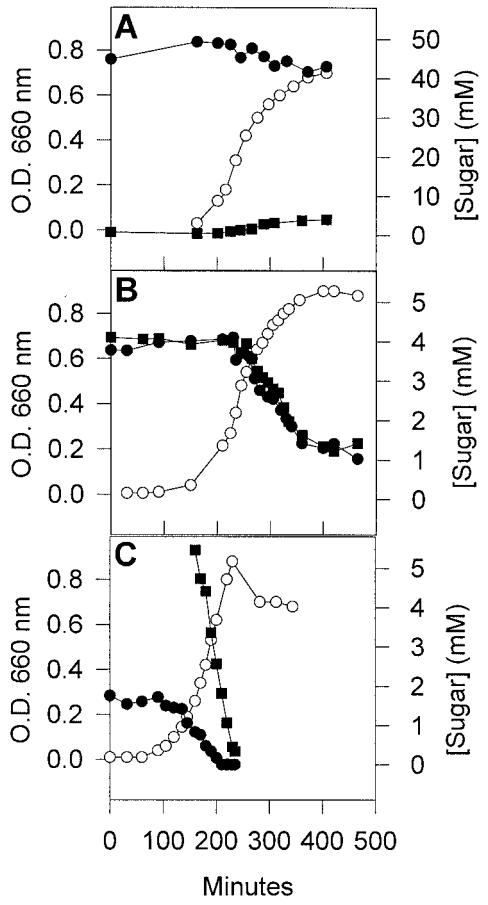

FIG. 1. Growth of *S. salivarius* on lactose and in a mixture of lactose and galactose. (A) Cells were grown at 37°C in M17 medium containing 50 mM lactose. Symbols: ○, growth; ● and ■, concentrations of lactose and galactose, respectively, in the medium. (B) *S. salivarius* was grown in a medium containing 5 mM lactose and 5 mM galactose. The symbols are as indicated in panel A. (C) An overnight culture of *S. salivarius* was used to inoculate a medium containing ca. 2 mM lactose. When the culture reached mid-log phase, the medium was supplemented with 5 mM galactose. The symbols are as indicated in panel A.

these changes prevented phosphorylation of His_{552}, we overproduced *S. salivarius* His_6-IIA^{LacS} in *E. coli*, purified it, and carried out phosphorylation tests with His_6-HPr(His~P) and His_6-HPr(Ser-P)(His~P).

Phosphorylation of *S. salivarius* His_6-IIA^{LacS} by His_6-HPr(His~P). The 3′ end of *S. salivarius lacS*, which codes for IIA^{LacS}, was expressed in *E. coli* BL21(DE3) as described in Materials and Methods. The purified protein migrated electrophoretically as a protein with a molecular mass of ~21,000 Da, which was close to the molecular mass calculated from the translated amino acid sequence (19,755 Da). The purified protein was used to test the phosphorylation of His_{552} by *S. salivarius* His_6-HPr(His~P). Incubating His_6-EI with [^{32}P]PEP resulted in the autophosphorylation of the recombinant enzyme (Fig. 2A, lane 3), a phenomenon that was not observed with His_6-IIA^{LacS} or His_6-HPr (Fig. 2A, lanes 1 and 2). Incubating His_6-EI and His_6-IIA^{LacS} with labeled PEP resulted in the phosphorylation of single protein corresponding to EI (not shown), whereas incubating His_6-EI and His_6-HPr with labeled PEP resulted in the phosphorylation of both proteins (Fig. 2A, lane 4). These results indicated that (i) the His-tag added to recombinant EI and HPr did not interfere with their capacity to receive and transfer a phosphate group and (ii) His_6-IIA^{LacS} could not be phosphorylated at the expense of PEP or His_6-EI(His~P). We then incubated His_6-EI, His_6-HPr, His_6-IIA^{LacS}, and [^{32}P]PEP together, removed samples at intervals, and analyzed the products by SDS-PAGE (Fig. 2B). The results clearly indicated that His_6-IIA^{LacS} was phosphorylated and that the amount of phosphorylated protein increased over time. When His_6-$IIA^{LacSH552R}$ was used instead of His_6-IIA^{LacS}, no phosphorylated His_6-$IIA^{LacSH552R}$ was detected on the autoradiogram (not shown), suggesting that His_6-HPr(His~P) phosphorylated the His_{552} of *S. salivarius* LacS.

Phosphorylation of *S. salivarius* His_6-IIA^{LacS} by His_6-HPr(Ser-P)(His~P). To determine whether His_6-IIA^{LacS} could be phosphorylated by His_6-HPr(Ser-P)(His~P), His_6-HPr(Ser-P) was first synthesized as described in Materials and Methods. The purity of His_6-HPr(Ser-P) was verified by native PAGE and silver nitrate staining. The results (data not shown) indicated that the preparation was free of His_6-HPr and HPrK/P. The absence of free His_6-HPr in the preparation was also demonstrated by incubating the purified preparation of His_6-HPr(Ser-P) with His_6-EI and [^{32}P]PEP and detecting the reaction products by autoradiography after separation by native PAGE. Only His_6-EI(His~P) and His_6-HPr(Ser-P)(His~P) were detected (data not shown). Analyses conducted with unlabeled PEP indicated that ca. 50% of the His_6-HPr(Ser-P) in the medium was transformed into His_6-HPr(Ser-P)(His~P) under the experimental conditions used. We thus incubated His_6-EI, His_6-HPr(Ser-P), and [^{32}P]PEP together to synthesize His_6-HPr(Ser-P)(His~^{32}P). Since only half of the His_6-HPr(Ser-P) was transformed into the doubly phosphorylated form under these conditions, we increased the concentration of His_6-HPr(Ser-P) twofold in the reaction medium to obtain a concentration of HPr(Ser-P)(His~P) similar to the concentration of HPr(His~P) (24 μM) that was used in the IIA^{LacS} phosphorylation experiments. His_6-IIA^{LacS} was then added to the reaction mixture. The results presented in Fig. 3A univocally indicate that His_6-IIA^{LacS} was phosphorylated by His_6-HPr(Ser-P)(His~P). No further increase in the amounts of His_6-IIA^{LacS}(His~P) was observed after 5 s, suggesting that the transfer of a phosphate group from HPr(Ser-P)(His~P) to IIA^{LacS} is a rapid process. The mutated protein His_6-$IIA^{LacSH552R}$ was not phosphorylated by His_6-HPr(Ser-P)(His~P) (not shown), suggesting that His_6-HPr(Ser-P)(His~P) and His_6-HPr(His~P) phosphorylated His_6-IIA^{LacS} on the same residue.

His_6-IIA^{LacS} could not be phosphorylated by His_{10}-$IIAB_L^{Man}$(His~P) or His_{10}-$IIAB_H^{Man}$(His~P). In a previous study, we demonstrated that *S. salivarius* P~$IIAB_L^{Man}$ and P~$IIAB_H^{Man}$ can transfer their phosphate groups to each other and possibly to other proteins (20). Circumstantial evidence also suggests that these PTS proteins control sugar metabolism by a mechanism that has yet to be characterized (5, 26, 38). We thus looked at whether these proteins could phosphorylate His_6-IIA^{LacS}. Purified His_{10}-$IIAB^{Man}$(His~P) proteins were first incubated with free HPr to determine whether the His tag interfered with their phosphotransfer capacity. As

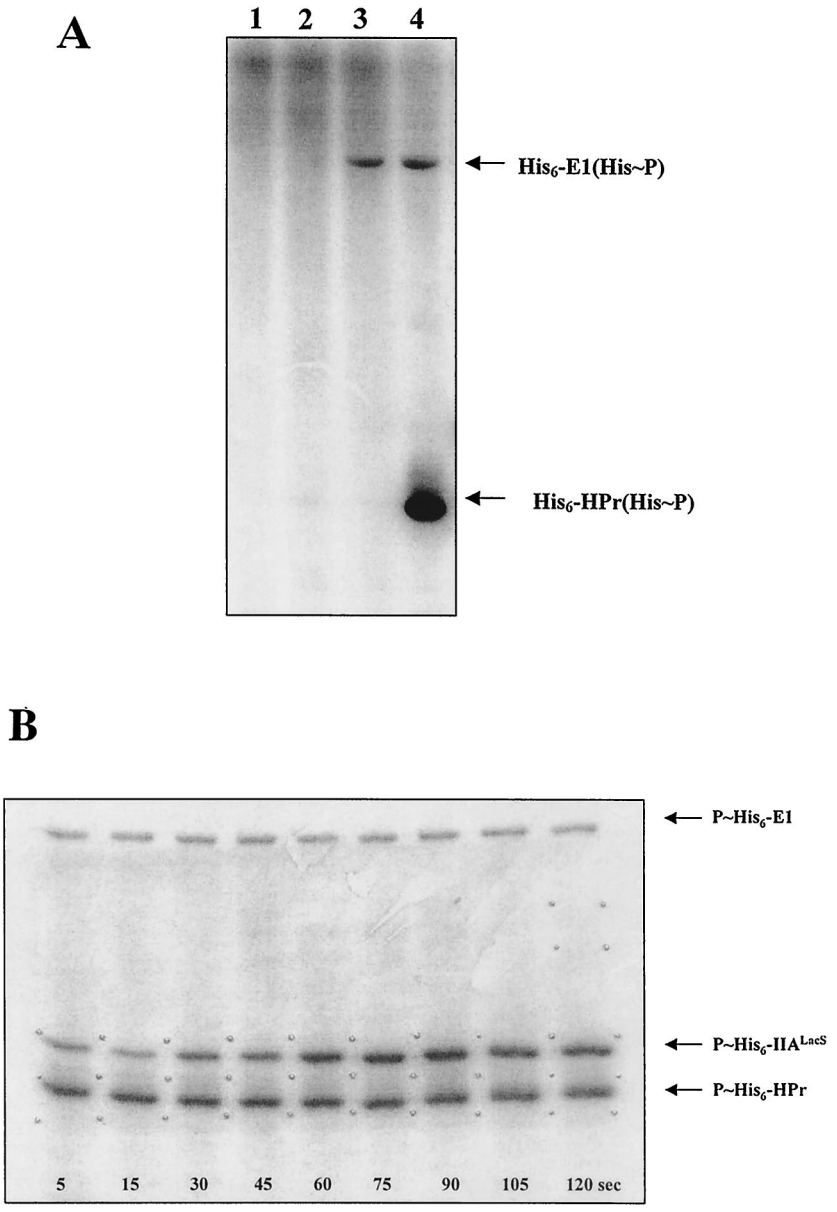

FIG. 2. PEP-dependent phosphorylation of His_6-IIA^{LacS} by His_6-HPr(His~P). The reactions were carried out in 50 mM Tris-acetate (pH 7.5) containing 1 mM DTT, 1 mM $MgCl_2$, 0.8 μM His_6-EI, 24 μM His_6-HPr, 5.8 μM His_6-IIA^{LacS}, and 1 mM [^{32}P]PEP (30 μCi/μmol). The reactions were stopped by adding an equal volume of a solution containing 180 mM Tris-HCl (pH 6.8), 200 mM SDS, 30% glycerol, 2 M β-mercaptoethanol, and 0.003% bromophenol blue. Proteins were separated by SDS-PAGE, and phosphoproteins were revealed by autoradiography. (A) Lanes: 1, control experiment conducted without EI and HPr; 2, control experiment conducted without EI and IIA^{LacS}; 3, control experiment conducted without HPr and IIALacS; 4, control experiment conducted without IIA^{LacS}. (B) PEP-dependent phosphorylation experiment conducted in a medium containing EI, HPr, and IIA^{LacS}. Samples were withdrawn at the intervals indicated on the autoradiogram.

shown in Fig. 3B (lane 1), His_{10}-$IIAB_H^{Man}$(His~P) could readily transfer its phosphate group to HPr. Identical results were obtained with His_{10}-$IIAB_L^{Man}$(His~P) (not shown). The results shown in Fig. 3B (lanes 2 and 3) indicate that His_{10}-$IIAB_L^{Man}$(His~P) and His_{10}-$IIAB_H^{Man}$(His~P) were unable to phosphorylate His_6-IIA^{LacS}.

Dephosphorylation of His_6-IIA^{LacS}(His~P) by HPr and HPr(Ser-P). To determine whether HPr(His~P) and HPr(Ser-P)(His~P) reversibly phosphorylated IIA^{LacS}, we incubated purified His_6-IIA^{LacS}(His~^{32}P) with HPr and HPr(Ser-P) under the conditions described in Materials and Methods and looked for the synthesis of HPr(His~^{32}P) and HPr(Ser-P)(His~^{32}P). As illustrated in Fig. 4, both HPr (lane 3) and HPr(Ser-P) (lane 2) could be phosphorylated by purified His_6-IIA^{LacS}(His~^{32}P).

Effect of LacS phosphorylation on the growth of *S. salivarius* on lactose and galactose. To determine whether LacS phosphorylation influences the growth of *S. salivarius* on lactose

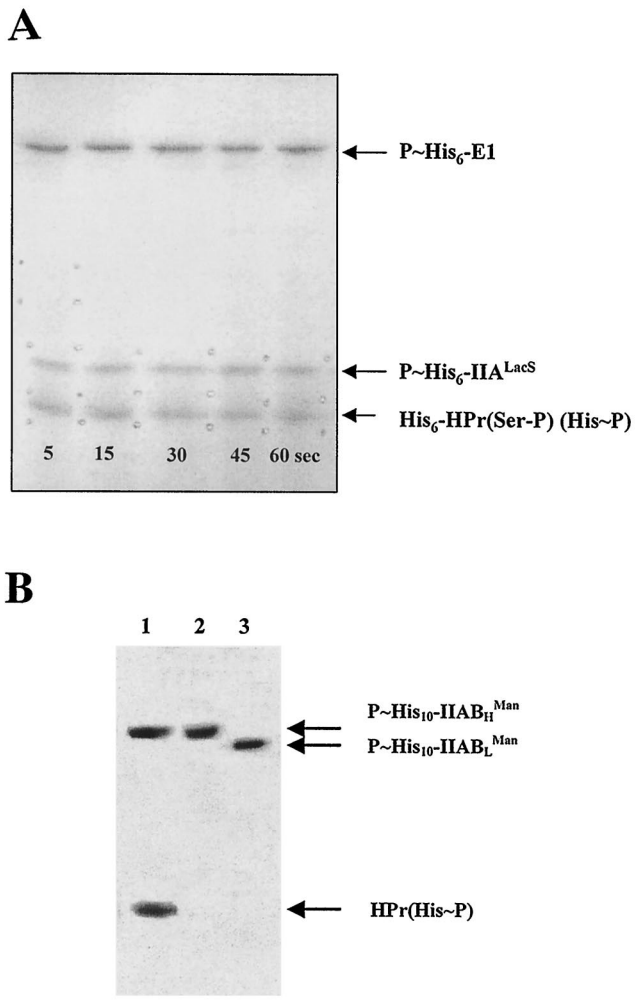

FIG. 3. Phosphorylation of His_6-IIA^{LacS} by His_6-HPr(Ser-P)(His~P) and by His_{10}-$IIAB_L^{Man}$(His~P) and His_{10}-$IIAB_H^{Man}$(His~P). (A) The synthesis of His_6-HPr(Ser-P)(His~^{32}P) is described in Materials and Methods. Phosphorylation of 5.8 μM His_6-IIA^{LacS} by ca. 20 μM His_6-HPr(Ser-P)(His~^{32}P) was conducted at 10°C. Samples were withdrawn at intervals, proteins were separated by SDS-PAGE, and phosphoproteins were revealed by autoradiography. (B) His_{10}-$IIAB_L^{Man}$(His~^{32}P) and His_{10}-$IIAB_H^{Man}$(His~^{32}P) were synthesized and purified as described in Materials and Methods. Phosphorylation of His_6-IIA^{LacS} by His_{10}-$IIAB_L^{Man}$(His~^{32}P) and His_{10}-$IIAB_H^{Man}$(His~^{32}P) was carried out in 10 mM HEPES (pH 7.5), containing 5 mM $MgCl_2$, either 2.8 μM His_{10}-$IIAB_L^{Man}$(His~^{32}P) or His_{10}-$IIAB_H^{Man}$(His~^{32}P), and 5.8 μM His_6-IIA^{LacS} in a total volume of 30 μl. The proteins were separated by SDS-PAGE and revealed by autoradiography. Lanes: 1, phosphorylated products resulting from the incubation of His_{10}-$IIAB_H^{Man}$(His~^{32}P) with His_6-HPr; 2, phosphorylated products resulting from the incubation of His_{10}-$IIAB_H^{Man}$(His~^{32}P) with His_6-IIA^{LacS}; 3, phosphorylated products resulting from the incubation of His_{10}-$IIAB_L^{Man}$(His~^{32}P) with His_6-IIA^{LacS}.

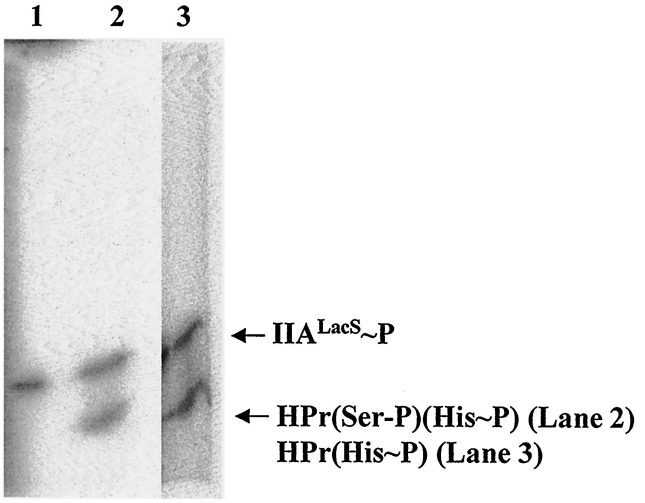

FIG. 4. Dephosphorylation of His_6-IIA^{LacS}(His~^{32}P) by HPr(Ser-P) and HPr. The synthesis and purification of His_6-IIA^{LacS}(His~^{32}P) is described in Materials and Methods. His_6-IIA^{LacS}(His~^{32}P) was dephosphorylated by HPr and HPr(Ser-P) in 50 mM Tris-acetate (pH 7.5) containing 1 mM DTT, 2 mM $MgCl_2$, and 20 μM HPr or HPr(Ser-P) in a total volume of 15 μl. After the mixture was incubated for 10 min at 37°C, His_6-IIA^{LacS}(His~^{32}P) was added to a final concentration of 2 μM, and the incubation was extended for 5 min. The proteins were separated by SDS-PAGE and revealed by autoradiography. Lanes: 1, purified His_6-IIA^{LacS}(His~^{32}P); 2, phosphorylated products resulting from the incubation of His_6-IIA^{LacS}(His~^{32}P) with HPr(Ser-P); 3, phosphorylated products resulting from the incubation of His_6-IIA^{LacS}(His~^{32}P) with HPr.

and galactose, two LacS substrates (13), we compared the growth properties of the wild-type and strain G71, an EI-negative mutant derived from *S. salivarius* ATCC 25975 (9). Since this mutant does not produce EI, growing cells do not contain HPr(His~P) or HPr(Ser-P)(His~P), which impedes LacS phosphorylation. The generation times of the wild-type and mutant strains on lactose and galactose are listed in Table 3. Strain G71 grew as well as the parental strain on 5.8 and 29 mM lactose. Moreover, like the wild-type strain (Fig. 1) (40), G71 did not release galactose into the external medium during growth on 5.8 mM (results not shown) and 58 mM lactose (Fig. 5A). Growth of the mutant strain was slightly reduced on galactose, with a generation time ~1.2-fold longer than that of the wild-type. The growth rates of the wild-type and mutant strains in a medium containing lactose and galactose also differed slightly. The growth of the wild-type strain under these conditions was as rapid as it was in a medium containing only lactose or galactose (about 27 min), whereas the doubling time of the mutant strain in a lactose-galactose mixture was 37 min, which corresponded to the generation time observed on galactose. Despite this difference in generation times, sugars were

TABLE 3. Generation times of *S. salivarius* ATCC 25975 and mutant G71

Sugar	Concn (mM)	Mean generation time[a] (min) ± SE	
		Parental strain	Mutant G71
Lactose	5.8	29 ± 1	28 ± 1
Lactose	29	28 ± 2	30 ± 2
Galactose	11	30 ± 1	36 ± 2
Galactose	55	32 ± 1	40 ± 3

[a] Growth was monitored by determining the optical density at 660 nm. Generation times were calculated for cultures in the exponential growth phase by plotting the logarithm of the optical density at 660 nm as a function of time. Values represent the means of eight separate experiments.

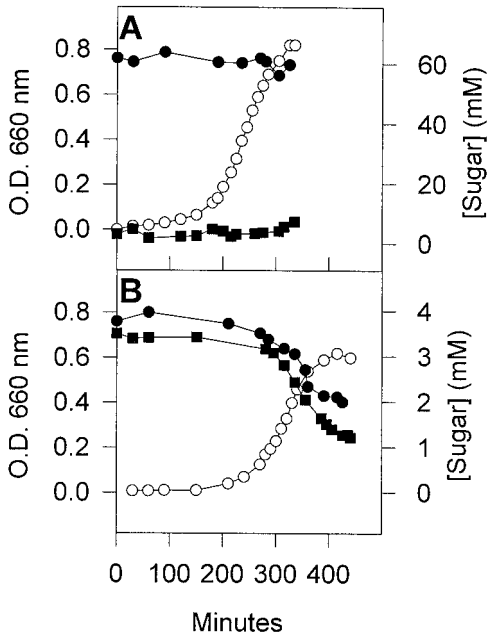

FIG. 5. Growth of mutant G71on lactose and in a mixture of lactose and galactose. (A) Cells were grown at 37°C in M17 medium containing 58 mM lactose. Symbols: ○, growth; ● and ■, concentrations of lactose and galactose, respectively, in the medium. (B) Mutant G71 was grown in a medium containing 4 mM lactose and 4 mM galactose. The symbols are as indicated in panel A.

consumed concomitantly by both strains during growth under these conditions (Fig. 1B and 5B).

DISCUSSION

Both *S. salivarius* and *S. thermophilus*, two closely phylogenetically related bacteria, take up lactose via a non-PTS transporter called LacS and hydrolyze intracellular lactose into glucose and galactose via a β-galactosidase (13, 14, 40). *S. thermophilus* LacS is a member of a subgroup of the galactoside-pentose-hexuronide family of transporters. These proteins have a C-terminal domain that shares significant amino acid sequence identity with PTS IIA domains, which are phosphorylated on a histidine residue by HPr(His~P) (25). Studies of lactose transport by *S. thermophilus* have provided compelling evidence that phosphorylation of LacS at His_{552} by HPr(His~P) stimulates lactose-galactose exchange by LacS (11, 12, 17, 41), resulting in galactose accumulation in the medium during growth on lactose. *S. salivarius* LacS shares 95% identity with the LacS from *S. thermophilus* SMQ-301 over the total length of the protein (40). The results reported here showed that *S. salivarius* LacS possessed a IIA domain with high levels of identity with IIA^{LacS} from various *S. thermophilus* strains. However, unlike *S. thermophilus*, *S. salivarius* did not accumulate galactose into the medium during growth on lactose and readily metabolized galactose, even in the presence of lactose (40) (Fig. 1). This raised the question of whether *S. salivarius* LacS was phosphorylated and, if so, what was the effect on growth at the expense of lactose and galactose.

Phosphorylation experiments conducted in vitro with purified proteins univocally showed that *S. salivarius* IIA^{LacS} could be reversibly phosphorylated by HPr(His~P) on residue His_{552}. *S. salivarius* cells contain considerable amounts of HPr(His~P) under conditions of limited growth and during the stationary growth phase, whereas this form of HPr is barely detectable in rapidly growing cells (32, 37). Thus, phosphorylation of LacS in vivo by HPr(His~P) obviously occurs mainly under conditions of restricted growth. Does this mean that *S. salivarius* LacS is weakly or not phosphorylated in rapidly growing cells? *S. salivarius* synthesizes at least two IIAB PTS proteins, $IIAB_L^{Man}$ and $IIAB_H^{Man}$, which catalyze interpeptide phosphotransfer and possibly phosphorylate other cellular proteins (20). Moreover, during the exponential phase of growth, *S. salivarius* synthesizes large amounts of the doubly phosphorylated form of HPr, HPr(Ser-P)(His~P), which accounts for approximately half of total cellular HPr (10, 22, 37). We thus looked at whether these proteins could phosphorylate IIA^{LacS}. Our results indicated that neither $P\sim IIAB_L^{Man}$ nor $P\sim IIAB_H^{Man}$ was able to transfer a phosphate group to LacS. However, the doubly phosphorylated form of HPr could readily phosphorylate LacS on His_{552}. These results suggest that a high proportion of LacS is in a phosphorylated state in rapidly growing *S. salivarius* cells.

It has been frequently reported that phosphorylation of HPr at Ser_{46} prevents phosphorylation at His_{15} and, conversely, phosphorylation at His_{15} impedes phosphorylation at Ser_{46}. Based on this observation and other biochemical studies, it is assumed that the phosphorylation of HPr at Ser_{46} by HPrK/P serves to reduce sugar uptake by the PTS (29). The fact, however, that growing streptococci contain considerable amounts of HPr(Ser-P)(His~P) suggests that phosphorylation of HPr at His_{15} or Ser_{46} does not prevent the synthesis of the doubly phosphorylated form of HPr and that the synthesis of HPr(Ser-P) does not interfere with the uptake of PTS sugars in streptococci (36). Our findings that HPr(Ser-P)(His~P) could be readily synthesized in vitro and was able to reversibly transfer a phosphate group to a IIA domain strengthen the view that the phosphorylation of HPr at Ser_{46} does not reduce PTS sugar transports in streptococci.

Since slowly and rapidly growing *S. salivarius* cells contain large amounts of a form of HPr that is able to phosphorylate LacS, the *S. salivarius* lactose permease most likely remains in a phosphorylated form. This contrasts with the results obtained from studies carried out with *S. thermophilus*, indicating that only 30% of LacS is phosphorylated in exponentially growing cells, whereas about two-thirds of the transporters are phosphorylated in early or late exponential cells (12). However, the results obtained with *S. thermophilus* cannot be compared to those from *S. salivarius* for at least two reasons. First, HPr(Ser-P)(His~P) in growing *S. thermophilus* cells does not exceed 5% of the total HPr (12), which is nearly 10 times lower than the levels in *S. salivarius*. Second, the levels of phosphorylated LacS in *S. thermophilus* were determined by using a strain in which the chromosomal *lacS* was deleted and which contained a plasmid bearing a copy of *lacS* under the control of its own promoter. Consequently, LacS levels during the exponential growth phase are 40-fold higher in the transformed strain than in the wild-type strain. This difference drops to sevenfold in early- and late-exponential-phase cells. Since the amounts of

HPr should be the same in the engineered and wild-type strains and do not change as a function of the growth phase (12), the ratios of HPr(Ser-P)(His~P)/LacS and HPr(His~P)/LacS in the engineered strain differ considerably from those in the wild-type cells. Thus, the amount of phosphorylated LacS in the engineered strain is likely different from that in the wild-type strain.

To determine the effect of *S. salivarius* LacS phosphorylation on the ability of cells to metabolize lactose and galactose, we studied the growth of strain G71, an EI-negative mutant derived from *S. salivarius* ATCC 25975 (9). Since this strain does not synthesize HPr(His~P) or HPr(Ser-P)(His~P), LacS should remain permanently unphosphorylated. Our results revealed that the wild-type and mutant strains had identical generation times on lactose and that neither expelled galactose during growth on lactose. Moreover, both strains metabolized lactose and galactose concomitantly when grown in a medium containing both sugars. These results suggested that *S. salivarius* LacS phosphorylation was not involved in the rate of growth on lactose, did not promote a discernible LacS-mediated lactose-galactose exchange, and did not change the ability of the transporter to transport galactose and lactose at the same time. We did observe, however, that the mutant strain grew slightly more slowly than the wild-type strain on galactose. This effect may result from the absence of LacS phosphorylation but may also result from other cellular perturbations caused by a modification in the relative proportion of the different forms of HPr in the EI-negative mutant. For instance, change in the intracellular amount of HPr(Ser-P) could affect transcription of genes under the control of the complex CcpA-HPr(Ser-P) (7, 13, 29) and the activity of permeases controlled by HPr(Ser-P) (29, 42). Although there is no direct evidence that *S. salivarius* LacS is regulated by HPr(Ser-P), it was demonstrated that the I47T substitution in *S. salivarius* HPr inhibits the preferential metabolism of glucose and fructose over lactose (10), indicating that somehow HPr is involved in the regulation of LacS. Moreover, a CcpA binding site (*cre* sequence) has been identified in the promoter region of the *S. salivarius gal* operon (40), and reduction in the levels of intracellular HPr by a factor of 3 interferes with expression of the *gal* operon (33). Lastly, we cannot rule out that *S. salivarius* possesses a second, as-yet-unidentified galactose transporter that would allow growth of mutant G71 on galactose. Thus, the small increase in the generation time on galactose observed with mutant G71 may result from several factors.

In conclusion, *S. salivarius* LacS could be readily phosphorylated on His_{552} by HPr(His~P), which is abundant in cells under conditions of energy privation, and by HPr(Ser-P)(His~P), which is synthesized in large amounts when energy sources are plentiful. The role of this phosphorylation remains unclear but did not seem to be related to galactose-lactose exchange and did not affect growth on lactose.

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