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Geertsma, Eric R.; Duurkens, Ria H.; Poolman, Berend

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The Activity of the Lactose Transporter from *Streptococcus thermophilus* Is Increased by Phosphorylated IIA and the Action of β-Galactosidase†

Eric R. Geertsma, Ria H. Duurkens, and Bert Poolman*

Department of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG, Groningen, The Netherlands

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ABSTRACT: The metabolism of lactose by *Streptococcus thermophilus* is highly regulated, allowing the bacterium to prefer lactose over glucose as main source of carbon and energy. In vitro analysis of the enzymes involved in transport and hydrolysis of lactose showed that the transport reaction benefits from the hydrolysis of lactose at the trans side of the membrane. Furthermore, the activity of LacS is modulated by PEP-dependent phosphorylation of the IIA domain via the general energy coupling proteins of the PTS, Enzyme I and HPr. To determine whether unphosphorylated LacS-IIA inhibited, or the phosphorylated form stimulated lactose counterflow, a LacS-IIA truncation mutant of LacS was constructed. Detailed analyses of transport in whole cells and in proteoliposomes indicated that unphosphorylated LacS-IIA does not functionally interact with the carrier domain. Instead, interaction of the phosphorylated form of LacS-IIA with the carrier stimulates lactose counterflow transport. The proposed mode of regulation thus proceeds via a mechanism opposite to the inducer exclusion type of regulation in Gram-negative bacteria, where transporters are inhibited by binding of the unphosphorylated form of IIA^{Glc}.

The physiology of *Streptococcus thermophilus* is highly adapted to living in milk and utilization of the main milk carbohydrate lactose. The completion of the genome sequence revealed that many genes involved in carbohydrate uptake and utilization are nonfunctional pseudogenes, in agreement with the low variety of carbon sources in milk. Furthermore, it showed that the lactose:proton symporter LacS, absent in most other streptococci, is the only secondary sugar transporter in *S. thermophilus* (*1*). The disaccharide lactose can be used by *S. thermophilus* as primary carbon and energy source. Lactose is internalized via LacS (*2*) and hydrolyzed internally by β-galactosidase to glucose and galactose. In contrast to the glucose moiety, galactose is not metabolized by most *S. thermophilus* strains and excreted via LacS (*3*). LacS has a higher affinity sugar binding at the cytoplasmic than at the extracellular side. Furthermore, the protein has a preference for galactose over lactose (*4*) which is consistent with a proton-neutral exchange of external lactose for internal galactose in vivo. This enables LacS to function as a net importer of the glucose moiety of lactose.

LacS consists of a membrane-embedded carrier domain and a hydrophilic IIA domain, residing at the cytoplasmic face of the membrane (*5*). The carrier domain catalyses the actual translocation reaction and forms a dimeric structure with subunits functionally interacting in the membrane (*6*−*9*). The IIA-domain is homologous to IIA^{Glc}-like domains of the PEP-PTS[1] system (*5*). LacS-IIA is not essential for transport but serves a regulatory role (*10*).

To ensure efficient metabolism of lactose, the rate of lactose uptake and hydrolysis are regulated at the level of biosynthesis and activity. The phosphoryl transfer protein HPr plays a central role in regulating LacS expression and activity. The serine-phoshorylated form of HPr, HPr(Ser-P), acts as a corepressor of CcpA and controls transcriptional repression of *lacSZ* (*11*), whereas the histidine-phosphorylated form of HPr, HPr(His∼P), phosphorylates the hydrophilic IIA domain of LacS and thereby affects the transport (*12*). The regulation of transport by the phosphorylation state of LacS-IIA contributes to a fast response of the cell to alterations in the external lactose concentration. Already after mid-exponential phase of growth, an increase in the level of HPr(His∼P) and a decrease in HPr(Ser-P) result in an increased transport activity and an increase in the biosynthesis of LacS, thereby compensating for the decrease of substrate (lactose) and increase of inhibitory end-product (galactose) in the medium (*11*).

In Gram-negative bacteria, the activity of several non-PTS carbohydrate transporters is regulated by the phosphorylation state of IIA^{Glc} via the inducer exclusion mechanism (*13*). In the presence of a PTS substrate, IIA^{Glc} becomes dephosphorylated due to the transfer of the phosphoryl group to the PTS substrate. This dephosphorylated form of *Escherichia coli* IIA^{Glc} interacts directly with its targets, among which are the melibiose permease MelB (*14*), the lactose transporter LacY (*15*, *16*), the raffinose permease RafB (*17*), the glycerol kinase (*18*), and the ATPase MalK of the maltose ABC-

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* To whom correspondence should be addressed. Phone, +31 50 363 4190; fax, +31 50 363 4165; e-mail, b.poolman@rug.nl.

[1] Abbreviations: PEP-PTS, phospho-enolpyruvate phosphotransferase system; GFP, green fluorescent protein; EI, enzyme I; IPTG, isopropyl-β-D-thiogalactopyranoside; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; Δ*p*, proton motive force; Egg PC, L-α-phosphatidylcholine from egg yolk.

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transporter (*19*), thereby decreasing the rates of uptake of the respective sugars.

Here, we demonstrate in a proteoliposomal system that β-galactosidase increases the efficiency of transport by hydrolyzing lactose and making galactose available for the exchange reaction, rather than by physical interactions between LacS and β-galactosidase. Furthermore, we show that regulation of transport by HPr(His∼P) involves interaction of the phosphorylated form of LacS-IIA with the carrier domain. This regulatory mechanism is opposite to the inducer exclusion type of regulation in Gram-negative bacteria.

EXPERIMENTAL PROCEDURES

Bacterial Strains. E. coli MC1061 (*20*) and NM522/pAG3 (*21*) were cultivated at 37 °C on Luria Broth under vigorous aeration. When appropriate, the medium was supplemented with 50 μg/mL ampicillin or 50 μg/mL carbenicillin. *S. thermophilus* ST11(ΔlacS)/pGKHis (*10*, *22*) was grown semianaerobically on Elliker Broth (*23*) at 42 °C supplemented with 5 μg/mL erythromycin, 1% lactose, and 0.5% beef extract.

Plasmid Constructions. DNA manipulations were done according to standard protocols. The Quikchange mutagenesis kit (Stratagene) was used to introduce site-directed mutations. Chromosomal DNA from *S. thermophilus* A147 was extracted as described (*24*). All mutants were verified by DNA sequencing.

1. pBADlacSC320A. By substituting the Pro codon CCA for CCT, the internal *NcoI* site in *lacS(C320A)* on pSKE8EhisC320A (*25*) was removed, yielding pSKE8EhisC320ANL. The *AatII*−*XbaI lacS*-fragment of pSKE8N (*22*) was replaced by the *NcoI*-less *AatII*−*XbaI lacS(C320A)*-fragment from pSKE8EhisC320ANL, yielding pNlacSC320Ahis. The *NcoI*−*XbaI* fragment of pNlacSC320Ahis was transferred to pBAD/Myc-His B (Invitrogen), yielding pBADlacSC320A.

2. pSKlacSC320AΔIIA. The six nucleotides 3′ of the codon for Glu-474 in *lacS(C320A)* on pSKE8EhisC320A were replaced by a *BamHI* site, yielding pSKE8EhisC320A-*BamHI*-IIA. A 368 bp *BamHI*−*XbaI* fragment coding for a Factor Xa cleavage site and a hexa-His-tag was derived from pNZOpuAAHis (*26*) and ligated into the *BamHI*−*XbaI*-digested vector, yielding pSKlacSC320AΔIIA.

3. pBADlacSC320AΔIIA. The *AatII*−*XbaI* fragment of pBADlacSC320A was exchanged for the *AatII*−*XbaI* fragment of pSKlacSC320AΔIIA, yielding pBADlacSC320AΔIIA.

4. pBADLacSC320AΔIIA-GFP. The 745 bp *BclI*−*XbaI* fragment of pNZOpuR-GFPuv (S. A. Henstra, unpublished data), containing the gene for the enhanced form of the *Aequorea victoria* green fluorescent protein (GFP), optimized for expression in *E. coli* (*27*), was ligated into *BclI*−*XbaI*-restricted pBADsub1C320A (*9*), yielding pBADLacSC-320AΔIIA-GFP containing an in-frame fusion of *GFP* to *lacS*Δ*IIA*.

5. pETbGalCHis. The *lacZ* gene was amplified by the Expand High Fidelity polymerase blend (Roche) using chromosomal DNA from *S. thermophilus* A147 and the primers 5′ATATATCCATGGGGAACATGACTGAAAA-AATTCAAACTTA (forward) and 5′TATATAGGATC-CATTTAGTGGTTCAATCATGAAGCTTAAT (reverse). The 3.1 kb product was digested by *NcoI* and *BamHI* and ligated into *NcoI*−*BamHI*-digested pET324 (*28*), yielding pETbGalCHis.

6. pETbGalCHisE547Q. The mutant E547Q allele was obtained by replacing the Glu-codon GAA for the Gln-codon CAA, using the Quikchange mutagenesis kit and pETbGal-CHis as template.

Membrane Vesicle Preparation. E. coli MC1061 cells harboring pSKlacSC320AΔIIA or pSKE8EhisC320A were ruptured by three passages through a French pressure cell at 10 000 psi, and the inside-out membrane vesicles were collected as described (*9*). Membrane vesicles were resuspended in 50 mM KPi, pH 7, frozen in liquid nitrogen, and stored at −80 °C. The protein concentration was determined using the DC Protein Assay (Bio-Rad).

Protein Purification. All steps during the purification of the proteins were performed at 4 °C unless indicated otherwise.

1. Enzyme I. Purification of *Bacillus subtilis* Enzyme I from *E. coli* NM522/pAG3 was done as described (*12*), with some modifications. Briefly, cells were washed, resuspended with 50 mM NaPi, pH 7, supplemented with 10% glycerol, 5 mM $MgSO_4$, and 0.1 mg/mL RNase and DNase, and lysed by three passages through a French pressure cell as described under Membrane Vesicle Preparation. Whole cells and membrane fractions were removed by centrifugation (184 000*g*, 1 h), the supernatant was supplemented with 5 mM β-mercaptoethanol, 100 mM NaCl, and 15 mM imidazole, and the pH was adjusted to 8 with K_2HPO_4. Next, the cytosolic fraction was mixed with Ni−NTA resin, washed with 50 mM KPi, 100 mM NaCl, 10% glycerol, and 5 mM β-mercaptoethanol (buffer A), pH 8, supplemented with 15 mM imidazole, and incubated for 1 h with continuous mixing. The column was drained, washed with 10 vol buffer A, pH 8, plus 15 mM imidazole and 10 vol buffer A, pH 8, plus 25 mM imidazole and eluted with buffer A, pH 7, plus 200 mM imidazole. Purified EI was extensively dialyzed against 50 mM Tris, pH 7.4, plus 3 mM dithiothreitol, and subsequently aliquoted and stored at −80 °C. The specific phosphorylation activity of Enzyme I was determined in a spectroscopic assay in which the formation of pyruvate from PEP upon phosphorylation of EI was coupled to the oxidation of NADH to lactate by lactate-dehydrogenase (*11*, *29*).

2. HPr. S. thermophilus ST11(ΔlacS)/pGKHis cells were washed with 20 mM Tris-Cl, pH 8.5, resuspended to OD_{660} = 75, and stored at −80 °C. To maximize the yield of HPr, cells were lysed by osmotic cell lysis (*2*) and subsequent three passages through a pressure cell at 1000 bar (*30*). The cytosolic fraction, obtained after removal of cell debris and membranes by centrifugation (184 000*g*, 1 h), was extensively dialyzed (membrane cutoff of 3.5 kDa) to decrease the K_2SO_4 concentration to below 1 mM. A white precipitate that appeared was removed by centrifugation. The bulk of the lysozyme was removed by three subsequent incubations of the supernatant with 20 mL of SP-sepharose for 1 h while stirring. The filtrate was loaded onto a Q-sepharose column (2.5 cm × 30 cm), washed with 20 mM Tris-Cl, pH 8.5, and eluted by supplementing the wash solution with 80 mM NaCl. Peak fractions were pooled and concentrated on an Amicon cell with a YM3.5 membrane. Purified HPr was aliquoted and stored at −80 °C. The specific phosphorylation activity of HPr was determined in a spectroscopic assay as described for EI.

3. LacS. LacS derivatives were purified from *E. coli* MC1061 membrane vesicles as described (*8*), but we stress that after solubilization the insoluble material was removed by centrifugation (15 min, 267 000*g*).

4. β-GalactosidaseSt. MC1061 cells containing either pETbGalCHis or pETbGalNHis were induced at OD_{660} = 0.8 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and growth was continued for 2 h. Cells were pelleted and washed with 50 mM KPi, pH 8.0, and resuspended in 50 mM KPi, pH 8.0, plus 1 mM $MgCl_2$ and tracer amounts of RNase and DNase. Cells were lysed by three passages through a French pressure cell at 10 000 psi and centrifuged for 10 min at 10 000*g*. The supernatant was centrifuged for 1 h at 184 000*g*, and its supernatant was supplemented with 10% glycerol and stored at −20 °C until purification.

Aliquots of 2 mL were supplemented with 200 mM NaCl and 5 mM imidazole, pH 8.0, and mixed for 1 h with 1 mL of MilliQ-washed Ni−NTA resin. The column was drained, washed with 10 vol of 50 mM KPi, pH 8.0, 200 mM NaCl, and 10% glycerol (buffer A) plus 5 mM imidazole and 10 vol of buffer A plus 10 mM imidazole. Next, the protein was eluted with 50 mM KPi, pH 7.0, 200 mM NaCl, and 10% glycerol plus 200 mM imidazole. Peak fractions were pooled, dialyzed against 50 mM KPi, pH 7.0, and 10% glycerol plus 5 mM $MgSO_4$, and aliquots were stored at −20 °C. The activity of β-galactosidaseSt was assayed at 37 °C in 50 mM KPi, pH 7.0, plus 5 mM $MgSO_4$ by following the hydrolysis rate of 4 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG) at A_{420} while stirring. β-Galactosidase from *E. coli* was purchased from Sigma Aldrich in purified form.

Whole Cell Transport Assays. 1. Cultivation. All transport assays were done with *E. coli* MC1061 cells. Cultivation was started with a 1% (v/v) inoculum of an overnight culture, and cells were grown until OD_{660} = 0.5−0.6 was reached. Cultures were induced with the appropriate amount of arabinose, and cultivation continued for 2.75 h. Cells were pelleted, washed twice with ice-cold 50 mM KPi, pH 7.1, plus 2 mM $MgSO_4$ (KPM pH 7.1), and resuspended to 36 mg protein/mL. Concentrated cell preparations were split and incubated at 4 °C overnight. As the overnight incubation did not compromise the integrity and metabolic activity of the cells, transport assays were performed the next day.

2. Transport Assays. Lactose transport was measured in dilute stirred cell suspensions at 30 °C unless indicated otherwise. The reaction was quenched at different time intervals by the addition of 2 mL of ice-cold 0.1 M LiCl, followed by rapid filtration on 0.45 μm nitrocellulose filters (Schleicher & Schuell Inc.). Reaction tubes and filters were washed with another 2 mL of ice-cold 0.1 M LiCl, and the radioactivity associated with the filter was determined by liquid scintillation counting.

3. Proton Motive Force (Δp)-Driven Lactose Uptake. Lactose accumulation in whole MC1061 cells was performed as described for DW2 cells (*8*).

4. Lactose Counterflow Transport. Cells were incubated overnight in the presence of 10 mM lactose. The next day, cells were washed with KPM, pH 7.7, supplemented with 10 mM lactose and concentrated to 50 mg protein/mL. Cells were de-energized by incubation with 50 μM SF6847 plus 30 mM NaN_3 for 2 h. Counterflow transport was started by 100-fold dilution of the cells into KPM, pH 7.7, yielding a final external ^{14}C-lactose concentration of 100 μM.

In Vitro Transport Assays. 1. Membrane Reconstitution. Membrane reconstitution of purified LacS and LacSΔIIA in liposomes, consisting of 3:1 ratio (w/w) of purified *E. coli* lipids and Egg PC, at a protein/lipid ratio of 141 pmol protein/mg lipid, was performed as described (*7*, *22*).

2. Orientation of Reconstituted LacSΔIIA. Proteoliposomes of LacSΔIIA were washed with 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, and 5 mM $CaCl_2$ (TNC), extruded through a 400 nm polycarbonate filter, and concentrated to 1 mg LacSΔIIA/mL by centrifugation (267 000*g*, 15 min, 10 °C). Aliquots (10 μL) were diluted to 20 μL, mixed with 0.5 μL of Factor Xa in 50% glycerol (Sigma), and incubated for 2−18 h at room temperature. The untreated sample was incubated without Factor Xa; the control sample was incubated with Factor Xa in the presence of 1% Triton X-100. To quench the reaction, PMSF was added to a final concentration of 1 mM. Subsequently, SDS−PAGE sample buffer with elevated SDS concentration (1.2%) was added, and the samples were stored at −20 °C. The increased SDS concentration prevented anomalous migration of the protein in the presence of a high lipid concentration. Subsequent SDS−PAGE, electroblotting, automated immunodecoration with a primary antibody directed against a hexa-His-tag (Amersham Pharmacia Biotech), and chemiluminescent detection was performed as described (*8*).

3. Transport Assays. Lactose transport in proteoliposomes was done as described for whole cells. Phosphorylation of membrane-reconstituted LacS was done as described (*12*).

4. Proton Motive Force (Δp)-Driven Lactose Uptake. Lactose transport driven by an artificial pH gradient and membrane potential was performed at 23 °C as described (*7*), except that the samples were diluted 100-fold. Briefly, proteoliposomes were washed and resuspended in 20 mM KPi, pH 7.0, and 100 mM potassium acetate plus 2 mM $MgSO_4$. Samples were frozen in liquid nitrogen and slowly thawed at room temperature. After extrusion through 400 nm polycarbonate filters to obtain predominantly unilamellar vesicles, proteoliposomes were concentrated to approximately 15 μM protein (1.1×10^2 mg lipid/mL). Transport was initiated by 100-fold dilution of aliquots into 120 mM Na−Pipes, pH 7.0, 2 mM $MgSO_4$, 0.5 μM valinomycin, and different concentrations of ^{14}C-lactose.

5. Lactose Counterflow Transport. Lactose counterflow was done as specified earlier (*4*). Briefly, proteoliposomes were washed and resuspended in 50 mM KPi, pH 7.0, and 2 mM $MgSO_4$ plus 10 mM lactose. Samples were frozen in liquid nitrogen and slowly thawed at room temperature. After extrusion through 400 nm polycarbonate filters, proteoliposomes were concentrated to approximately 15 μM protein. Transport was initiated by 100-fold dilution of aliquots into 50 mM KPi, pH 7.0, and 2 mM $MgSO_4$ plus different concentrations of ^{14}C-lactose.

6. Lactose Efflux Transport Assay. Proteoliposomes were washed and resuspended in 50 mM KPi, pH 7.0, and 5 mM $MgSO_4$ plus 5 mM lactose. Samples were frozen in liquid nitrogen and slowly thawed at room temperature. After extrusion through 400 nm polycarbonate filters, proteoliposomes were concentrated and equilibrated with 5 mM [D-glucose-1-^{14}C]lactose for at least 2 h. Transport was measured in stirred proteoliposome suspensions at 37 °C and initiated by 100-fold dilution of 20 μM LacS aliquots into 50 mM KPi, pH 7.0, and 2.5% glycerol plus 5 mM $MgSO_4$.

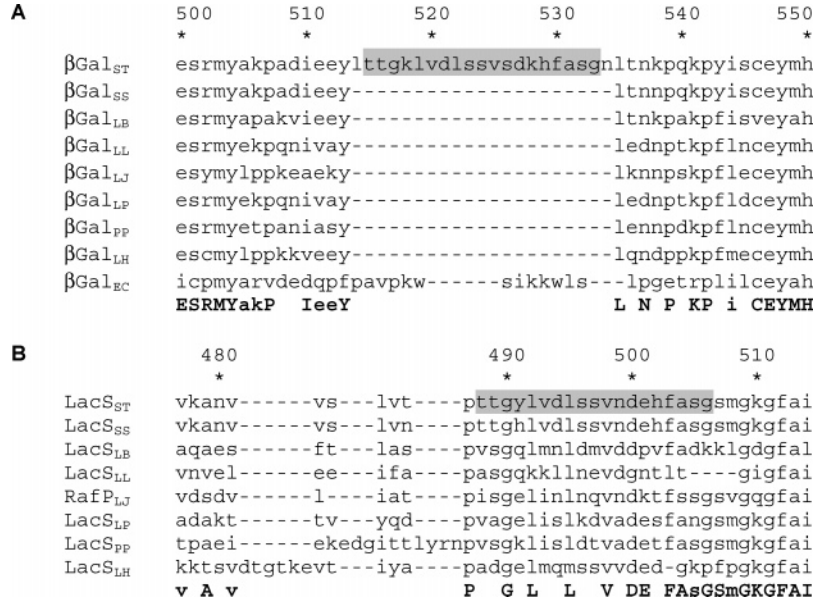

FIGURE 1: Amino acid sequence alignments of β-galactosidases and LacS homologues. The stretches of amino acids present in both β-galactosidase and LacS from *S. thermophilus* are marked gray. Panels A and B show multiple sequence alignments of β-galactosidase and LacS homologues, respectively. Residues are numbered based on the amino acid sequence of the *S. thermophilus* proteins. The consensus sequence is depicted in bold below each alignment. The subscripts ST, SS, LB, LL, LJ, LP, PP, LH, and EC refer to *Streptococcus thermophilus*, *Streptococcus salivarius*, *Lactobacillus bulgaricus*, *Leuconostoc lactis*, *Lactobacillus johnsonii*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Lactobacillus helveticus*, and *Escherichia coli*, respectively. The accession numbers for the shown sequences are (in the order shown) A49750, AAL67294, AAA25244, A42891, NP964710, NP786691, ZP00322673, AAU11509, and AAC73447 for the β-galactosidases and P23936, AAL67293, AAA25243, AAC44113, NP964277, NP786694, ZP00322671, and AAU11510 for the LacS homologues.

To measure lactose efflux in the presence of wild-type or inactive (E547Q) β-galactosidase, concentrated proteoliposomes were diluted in the same buffer plus 5 μM β-galactosidase. For the lactose exchange transport assay, the outside buffer was supplemented with 10 mM lactose. The reaction was quenched at different time intervals by the addition of 2 mL of ice-cold 0.1 M LiCl, followed by rapid filtration on 0.45 μm nitrocellulose filters (Schleicher & Schuell Inc.). Reaction tubes and filters were washed with another 2 mL of ice-cold 0.1 M LiCl, and the radioactivity associated with the filter was determined by liquid scintillation counting.

RESULTS

The Action of β-Galactosidase Increases the Rate of Lactose Transport. Indications for a physical interaction between β-galactosidaseSt and LacS came from the partial co-purification of β-galactosidaseSt with LacS and comigration of LacS with β-galactosidaseSt on Blue Native gels (unpublished observations). Additionally, both proteins were found to contain a remarkably similar stretch of 19 amino acids (TTG[K/Y]LVDLSSV[S/N]D[K/E]HFASG), absent in other *S. thermophilus* proteins. This stretch is moderately conserved in LacS homologues from other species (except the closely related *Streptococcus salivarius*; consensus motif xxGxLxxLxxVxDExFAsG) and completely absent in homologues of β-galactosidaseSt (Figure 1). On the basis of the 3D structures of the homologous *E. coli* enzymes IIA^{Glc} (*31*) and β-galactosidase^{Ec} (*32*), these stretches are most probably located on the surface of LacS and β-galactosidaseSt and could form a potential site of interaction. Here, we set out to determine whether β-galactosidaseSt and LacS interacted functionally.

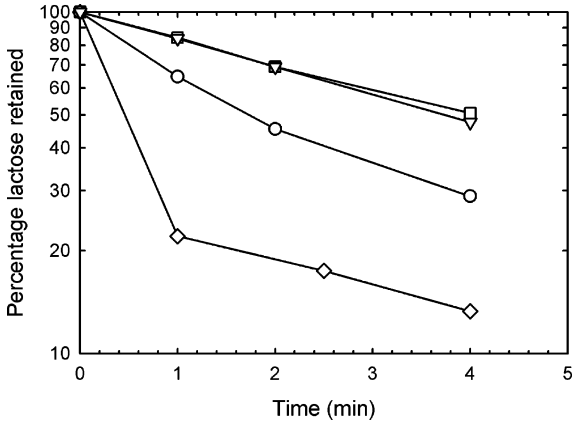

FIGURE 2: Effect of LacZ on lactose efflux by LacS. Proteoliposomes were equilibrated with 5 mM ^{14}C-lactose. Lactose efflux was initiated by 100-fold dilution into 50 mM KPi, pH 7.0, and 2.5% glycerol plus 5 mM $MgSO_4$, yielding a final LacS concentration of 0.2 μM (squares). Additionally, ^{14}C-lactose efflux was assayed in buffers supplemented with 10 mM lactose (diamonds), 5 μM LacZ (circles), or 5 μM LacZ(E547Q) (triangles). The presence of 5 μM LacZ yielded a 25-fold excess of LacZ over LacS. Representative curves are shown.

LacS was membrane-reconstituted with the cytoplasmic side facing the external medium. By loading LacS proteoliposomes with 5 mM D-(glucose-1-^{14}C)lactose and monitoring the LacS-mediated release of the radioisotope from the lumen of the proteoliposomes upon rapid dilution, the in vivo situation with respect to lactose concentrations and sugar flow was mimicked. Figure 2 shows that the rate of lactose exit was dependent on the composition of the external medium. Without lactose in the external medium, the ^{14}C-lactose exit was slow. In the presence of 10 mM nonisotopically labeled

lactose in the external medium, the rate of exit was greatly increased, which is consistent with earlier observations on lactose efflux and exchange (*4*).

To determine the role of β-galactosidase in LacS-mediated lactose transport, the *lacZ* gene from *S. thermophilus* A054 was cloned and equipped with a sequence encoding a C-terminal His_6-tag. The purified enzyme, designated β-galactosidaseSt, hydrolyzed the chromogenic substrate ONPG with a K_m^{App} of approximately 0.8 mM and a k_{cat} of 6.7×10^2 s^{-1}. As shown in Figure 2, the addition of 5 μM β-galactosidaseSt to the external medium (yielding a LacS/β-galactosidaseSt molar ratio of 1:25), resulted in an increase in the rate of lactose efflux. The hydrolysis of lactose by β-galactosidaseSt resulted in the formation of ^{14}C-glucose, a substrate not transported by LacS, and nonisotopically labeled galactose, a "high-affinity" substrate of LacS. The acceleration of transport was the result of the hydrolysis of ^{14}C-lactose by β-galactosidaseSt rather than a direct interaction between both proteins. The evidence for this notion is the following: (*i*) using the *E. coli* β-galactosidase^{Ec}, which is unlikely to interact with LacS, a similar increase in lactose efflux was observed (data not shown); (*ii*) addition of 5 μM β-galactosidaseSt(E547Q), a catalytically impaired mutant of β-galactosidaseSt, did not result in an increase in lactose efflux (Figure 2); and (*iii*) the percentages of lactose retained by the proteoliposomes after 3 min incubation were equal in the presence of 5 μM wild-type β-galactosidaseSt only or both 5 μM wild-type and 5 μM β-galactosidaseSt(E547Q) (data not shown).

Taken together, the data presented here suggest that lactose transport by LacS is not affected by specific interactions with β-galactosidaseSt but that the mere presence of lactose hydrolysis activity accelerates the rate of lactose efflux from proteoliposomes due to the production of the higher affinity countersubstrate galactose.

Deletion of LacS-IIA Differently Affects Lactose Counterflow and Δp*-Driven Lactose Uptake.* To specify the mode of regulation by LacS-IIA, a truncated version of Cys-less LacS was constructed, in which the C-terminal 160 amino acids, corresponding to the IIA domain, were deleted. The C-terminus of this construct, designated LacSΔIIA, still comprised the linker region connecting the carrier and LacS-IIA domain and, additionally, a hexa-His tag. LacSΔIIA, expressed in *E. coli* MC1061, migrated on SDS−PAGE at approximately 40 kDa, while LacS migrated around 55 kDa. Initial activity analyses were done in *E. coli* MC1061 in which the expression of LacS and derivatives was controlled by the AraC/P_{BAD} expression system.

The IIA domain of LacS, expressed as soluble protein (IIA^{LacS}), can be phoshorylated in vitro and in vivo by HPr-(His∼P) from *E. coli* (*33*), suggesting that the membrane-associated LacS-IIA domain might also be phosphorylated in *E. coli* cells. To determine whether the phosphorylation state of LacS is kept constant over a range of expression levels, and not affected by, for example, titrating out HPr (knowing that the phosphoryl transfer between HPr and LacS is extremely slow), we measured the initial uptake rates in cells induced with different concentrations of L-arabinose. Both lactose counterflow and Δp-driven lactose uptake (Figure 3) were measured (Figure 4), since these transport modes respond differently to LacS-IIA phosphorylation (*12*). For LacS, the ratio of the activities of both modes of transport

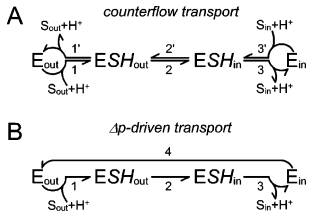

FIGURE 3: Schematic representation of counterflow and Δp-driven transport. *E* reflects the unliganded protein, *ESH* the fully liganded protein with both the substrate and the proton binding site occupied. *S* and *H* represent the substrate (a galactoside) and the proton, respectively. The subscripts *out* and *in* refer to the location of the binding sites, extracellular and cytoplasmic, respectively. In the wild-type protein, both the fully liganded and unliganded binding sites can reorient. (A) Lactose counterflow transport. A substrate is bound on the outside (step 1), the binding sites reorient (step 2), and the substrate is released on the inside (step 3). Next, another substrate is bound on the inside (step 3′), and reorientation to the extracellular site takes place (step 2′). Upon release of the substrate (step 1′), another round of translocation can start. Note that the counterflow mode of transport is overall an electroneutral process. The reorientation of the liganded carrier (steps 2 and 2′) is proposed to be accelerated upon phosphorylation of LacS-IIA and be responsible for the increased lactose counterflow rate of phosphorylated LacS. (B) Δp-Driven lactose transport. Similar steps (nos. 1, 2, and 3) as described for counterflow transport take place, but upon release of the substrate on the inside (step 3), substrate is not bound on the trans side. Rather, the unliganded binding site reorients to the outside (step 4) and initiates another round of translocation.

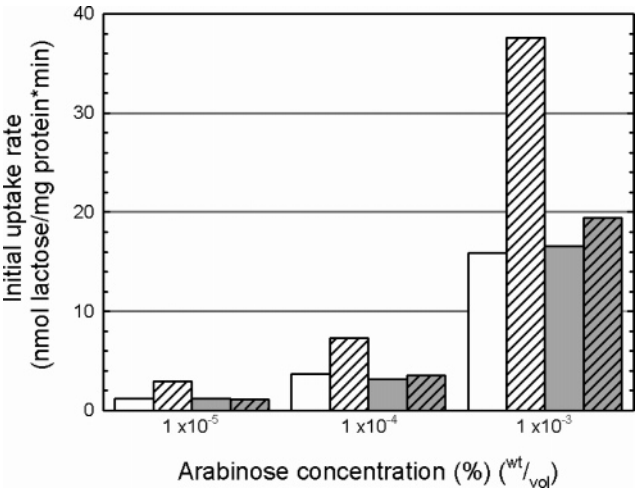

FIGURE 4: Initial rates of lactose transport by LacS and LacSΔIIA at different inducer concentrations. *E. coli* MC1061 cells were induced with 1×10^{-5}, 1×10^{-4}, or 1×10^{-3}% (w/v) of L-arabinose and cells were prepared as described under Experimental Procedures. White and gray bars represent initial uptake rates of LacS and LacSΔIIA, respectively. Open and hatched bars reflect Δp-driven and counterflow lactose transport, respectively.

was constant over a broad range of inducer concentrations, suggesting that the initial transport rates were only governed by the concentration of the LacS protein in the membrane. Also, the ratio between the initial rates of Δp-driven lactose transport and lactose counterflow by LacSΔIIA was constant in this inducer range but differed significantly from the ratio observed for LacS. The initial rates of Δp-driven lactose transport were equal for LacS and LacSΔIIA, but the initial rate of counterflow transport by LacSΔIIA was only half that of LacS. As lactose counterflow is more responsive to the phosphorylation state of LacS-IIA than Δp-driven lactose uptake (*12*), the deletion of LacS-IIA most likely decreases

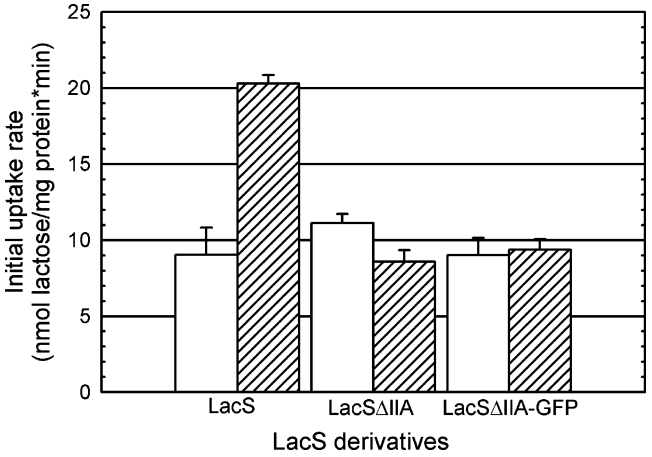

FIGURE 5: Initial rates of lactose transport by LacS, LacSΔIIA, and LacSΔIIA-GFP. *E. coli* MC1061 cells harboring different plasmids were cultivated, induced with 1×10^{-3}% (w/v) of L-arabinose, and prepared and assayed as described under Experimental Procedures. LacS is the parent protein and corresponds to LacS(C320A) with a C-terminal His-tag; LacSΔIIA is the 160 amino acid truncation mutant of LacS; LacSΔIIA-GFP consists of a C-terminal fusion of GFP to LacSΔIIA. Open and hatched bars represent Δ*p*-driven lactose transport and counterflow transport, respectively.

the initial rate of lactose counterflow, whereas it has no effect on Δ*p*-driven transport.

Substitution of the IIA Domain by GFP Differently Affects Lactose Counterflow and Δp-Driven Lactose Uptake. To exclude the possibility that the decreased rate of LacSΔIIA counterflow transport was caused by an aspecific global rather than regulatory effect of the deletion of the 17 kDa cytoplasmic domain, the 26 kDa *A. victoria* green fluorescent protein (GFP) was fused to the C-terminus of LacSΔIIA-(C320A). This 79 kDa protein, designated LacSΔIIA-GFP, was expressed and correctly inserted into the membrane as shown by fluorescent confocal imaging (unpublised data). The initial rates of counterflow and Δ*p*-driven lactose transport by LacSΔIIA-GFP were equal to LacSΔIIA (Figure 5), suggesting that the transport activity of the carrier domain is specifically affected by the removal of the IIA-domain.

LacSΔIIA Is Membrane-Reconstituted in the Inside-Out Orientation. To allow manipulation of the LacS-IIA phosphorylation state, further analyses were done in vitro. Since full-length LacS is reconstituted in liposomes in the inside-out orientation (*22*), and the affinities of the outward and inward facing lactose-binding sites differ approximately 1 order of magnitude, it was important to determine the orientation of membrane-reconstituted LacSΔIIA. LacSΔIIA was reconstituted as described (*7*, *22*), and the orientation in the membrane was assessed by determining the accessibility of the Factor Xa cleavage site, which precedes the C-terminal hexa-His tag of LacSΔIIA. Proteolysis by Factor Xa detaches the His-tag from the protein. Separation of the protein on SDS−PAGE and subsequent immunoblotting with anti-His antibody gives an estimate of the amount of inside-out-oriented protein. Equal amounts of protein were analyzed, and immunoblotting showed that the untreated protein had a high reactivity toward the anti-His antibody, whereas detergent-solubilized and Factor Xa-treated LacSΔIIA proteoliposomes gave no signal (Figure 6). The LacSΔIIA proteoliposomes, treated with Factor Xa only, showed very

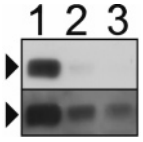

FIGURE 6: Immunoblots of Factor Xa-treated proteoliposomes harboring LacSΔIIA. Proteoliposomes containing LacSΔIIA were incubated for 2 h at room temperature in the absence (lane 1), in the presence of Factor Xa (lane 2), and in the presence of Factor Xa plus 1% Triton X-100 (lane 3). Arrowheads on the left side indicate the LacSΔIIA signal. The top and bottom box show immunoblots decorated with anti-His antibody illuminated for short (5 min) and prolonged periods (1 h) of time, respectively.

low reactivity with the antibody; only after long exposure, a faint band became visible (Figure 6, bottom box, lane 2). The reactivity of Factor Xa-treated LacSΔIIA in intact proteoliposomes was only a little more intense than detergent-solubilized and Factor Xa-treated LacSΔIIA, indicating that LacSΔIIA was incorporated in the lipid vesicles predominantly in an inside-out orientation, similar to the full-length LacS protein, allowing a direct comparison of both species.

The Transport Kinetics of LacSΔIIA Mimic That of Dephosphorylated LacS. To avoid differences in reconstitution efficiency due to variations in detergent concentration, purified LacS and LacSΔIIA were brought to the same molar concentration before being mixed with the Triton X-100-titrated liposomes. For transport driven by the proton motive force (Δ*p*), the proteoliposomes were equilibrated with 20 mM KPi, pH 7.0, and 100 mM potassium acetate plus 2 mM $MgSO_4$. The artificial Δ*p* was formed by diluting the proteoliposomes at time zero 100-fold into 120 mM Na−Pipes, pH 7.0, and 2 mM $MgSO_4$, plus 0.5 μM valinomycin.

Purified and membrane-reconstituted LacS was in the dephosphorylated state since the addition of an excess of HPr from *S. thermophilus*, which accelerates IIA^{LacS} dephosphorylation (*33*), had no effect on counterflow and Δ*p*-driven lactose transport (results not shown). To phosphorylate LacS, proteoliposomes were incubated with purified EI from *B. subtilis* and HPr from *S. thermophilus* in the presence of PEP (*12*). In correspondence with earlier observations, Δ*p*-driven lactose transport was slightly inhibited in the phosphorylated full-length LacS protein (Figure 7A). As shown previously, this is caused by a small decrease in affinity (*12*). The rates of Δ*p*-driven lactose transport by LacSΔIIA fitted best with the activity of dephosphorylated LacS.

For lactose counterflow transport, the proteoliposomes were equilibrated with 10 mM lactose, concentrated, and diluted 100-fold into buffer containing tracer amounts of ^{14}C-lactose, yielding a final external lactose concentration of approximately 100 μM. In contrast to the small inhibitory effect of phosphorylation on Δ*p*-driven lactose transport, lactose counterflow was highly stimulated with phosphorylated LacS (Figure 7B). The kinetics of lactose counterflow transport by LacSΔIIA were similar to the kinetics of dephosphorylated LacS. Taken together, the combined data of both Δ*p*-driven lactose uptake and lactose counterflow convincingly demonstrate that LacSΔIIA mimics the transport characteristics of LacS in the dephosphorylated state.

DISCUSSION

S. thermophilus efficiently metabolizes lactose, which it prefers over glucose as main carbon source. Based on the

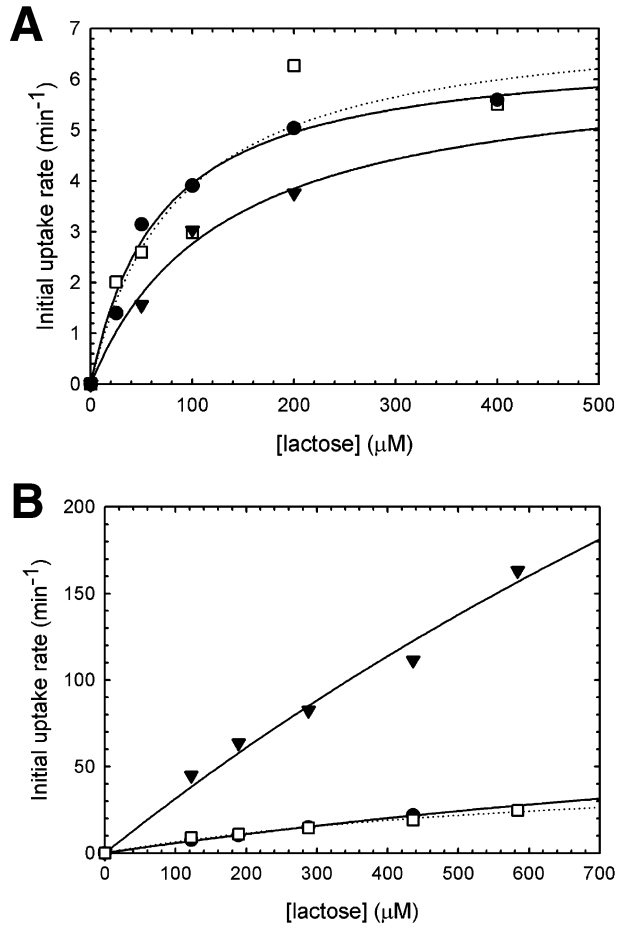

FIGURE 7: Lactose transport by LacSΔIIA and (un)phosphorylated LacS in proteoliposomes. Initial rates of Δ*p*-driven lactose transport (A) and lactose counterflow (B) were plotted and fitted to the Michaelis−Menten equation. Note that the transport rates are expressed as mol lactose internalized/(mol transporter·min) (min^{-1}). Black and white symbols represent transport rates of LacS and LacSΔIIA, respectively. Triangles and circles indicate phosphorylated and unphosphorylated LacS. Solid lines show the Michaelis−Menten fit of the LacS samples, while the fit for LacSΔIIA is represented by a dotted line.

partial copurification of β-galactosidaseSt and LacS, we determined whether functional interactions between both proteins played a role in the transport of lactose. In vitro analysis of the two component pathway did not confirm an association between β-galactosidaseSt and LacS. However, the data indicates that in vivo the sole presence of β-galactosidaseSt suffices to accelerate the rate of lactose import. By hydrolyzing lactose, β-galactosidaseSt makes galactose available for the exchange reaction and thereby indirectly increases the lactose uptake rate.

The lactose transport capacity in *S. thermophilus* is regulated at the transcriptional level by GalR and HPr(Ser-P)-mediated CcpA repression, and at the level of the LacS activity, by HPr(His∼P)- and possibly HPr(Ser-P)(His∼P)-mediated phosphorylation of the LacS-IIA domain. This regulatory network allows the tuning of transport to the metabolism of lactose and the energy status of the cell and avoids an overcapacity of transport, which can ultimately lead to decreased growth rates and lower cell density as has been show in a *ccpA* disruption strain (*34*).

In this study, the regulation of the lactose transport activity of the membrane-embedded carrier domain of LacS by the LacS-IIA domain was examined. Previous studies on the role of the IIA domain of LacS had already shown that (*i*) the LacS-IIA domain is not essential for translocation (*10*); (*ii*) LacS is phosphorylated on His-552 by either HPr(His∼P) or HPr(Ser-P)(His∼P) (*33*, *35*, *36*), whereas LacS is not affected by HPr(Ser-P) (*12*); and (*iii*) the LacS-IIA domain modulates the activity of the carrier domain. However, it was not established whether regulation took place via interaction of the carrier domain with phosphorylated LacS-IIA, dephosphorylated LacS-IIA, or both LacS-IIA species.

To determine which scenario was applicable, a C-terminal 160 amino acid truncation mutant, LacSΔIIA, was constructed. In *E. coli* MC1061, transport by LacSΔIIA was compared to LacS and LacSΔIIA-GFP. The use of LacSΔIIA-GFP was preferred over LacS-IIA mutants of His-552, because the phenotype of such IIA mutants might mimic that of phosphorylated IIA (*37*). Counterflow transport by LacS proceeded at approximately twice the rate of Δ*p*-driven lactose transport, while both LacSΔIIA and LacSΔIIA-GFP showed similar rates for both modes of transport. The activities of LacSΔIIA and LacSΔIIA-GFP were equal to Δ*p*-driven transport by LacS. Since both the LacS-IIA deletion and GFP-substitution mutant displayed similar transport characteristics, the presence or absence of a C-terminal soluble domain does not seem to affect the activity of the carrier domain. We therefore propose that the LacS-IIA domain must physically interact with the carrier domain in order to exert its regulatory role. Based on the in vitro transport data, showing that LacSΔIIA indeed mimics unphoshorylated LacS, the transport characteristics of LacS in *E. coli* cells suggest that the protein is phosphorylated under these conditions.

Conclusive evidence on the mechanism of regulation of LacS-IIA came from in vitro studies. Full-length LacS was membrane-reconstituted in a predominantly inside-out orientation. It has now been shown that the absence of the LacS-IIA domain yields proteoliposomes in which the carrier domain is in the same orientation as the full-length LacS protein, indicating that the orientation is not determined by the large hydrophilic LacS-IIA domain as suggested previously (*22*), but by the membrane-embedded carrier domain itself. Since the affinities of both binding sites of LacS differ approximately by 1 order of magnitude, the identical orientation of LacS and LacSΔIIA in proteoliposomes enabled a direct comparison of the activities of both proteins in vitro.

In vitro analyses of the effect of phosphorylation of LacS on the translocation of lactose indicated that the rate of Δ*p*-driven lactose transport of phosphorylated LacS is somewhat decreased compared to the unphosphorylated species. The V_{max} of lactose counterflow transport of phosphorylated LacS, in contrast, was increased 4−6 times. Both observations are in agreement with previous studies (*10*, *12*). The stimulation of lactose counterflow was explained by assuming an increase in the rate constants for the reorientation of the ternary enzyme−substrate−proton complex (Figure 3) upon phosphorylation (*12*). This isomerization of the ternary complex has been proposed to be the rate-limiting step in lactose counterflow transport (*38*). Although Δ*p*-driven lactose transport also comprises a reorientation step of the ternary complex, the V_{max} for this mode of transport was

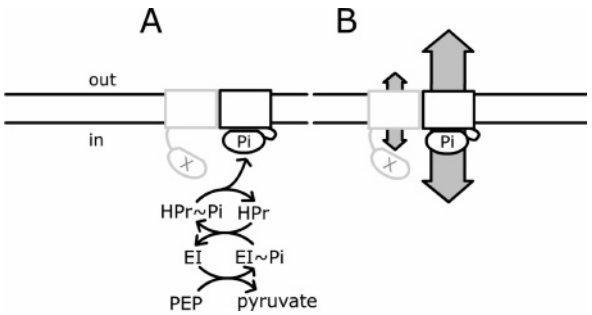

FIGURE 8: A model of the interactions between the carrier and IIA domain of LacS. The membrane-embedded carrier domain of LacS is represented by a white box to which the soluble IIA domain (represented by a white oval) is coupled via a flexible linker. The phosphorylation state of LacS-IIA is represented by X and Pi, reflecting the unphosphorylated and the phosphorylated state, respectively. The two-way arrows indicate lactose counterflow transport, and their size reflects the rate of transport. (A) Upon phosphorylation of the IIA domain by HPr(His∼P) or HPr(Ser-P)-(His∼P), the IIA domain interacts with the carrier domain; (B) this interaction accelerates the counterflow transport. The other subunit of the dimer (depicted in gray) is not affected by the opposing IIA domain. For clarity, only one phosphorylated subunit per dimer is shown.

unaffected, which agrees with the idea that this step is not rate-determining for Δ*p*-driven uptake.

The results obtained thus far are summarized in a working model (Figure 8). For both lactose counterflow and Δ*p*-driven lactose transport, the kinetics of translocation of unphosphorylated LacS and LacSΔIIA are very similar, suggesting that the LacS-IIA domain does not functionally interact with the carrier domain in its unphosphorylated state. The stimulation of lactose counterflow upon phosphorylation of LacS-IIA by HPr(His∼P) is most likely caused by a direct interaction of the phosphorylated IIA domain with the carrier domain. As the levels of the different HPr species depend on the strain and growth phase of the organism (*11*, *36*), these parameters will determine which of the two histidine-phosphorylated HPr species [HPr(His∼P) or HPr(Ser-P)-(His∼P)] will be most involved in the phosphorylation of LacS.

The regulation by LacS-IIA takes place intramolecularly as inferred from the analysis of tandem fusion proteins consisting of a LacSΔIIA subunit fused to the N-terminus of a LacS subunit (*9*). These covalent dimers contained only one LacS-IIA domain per dimer, and either the first or the second carrier domain was inactivated by mutation. Only tandem constructs in which the LacS-IIA domain was attached to a functional carrier showed an increase in counterflow transport relative to Δ*p*-driven transport, indicating that LacS-IIA regulation takes place within one subunit.

The proposed mode of regulation differs from the mechanism of inducer exclusion in *E. coli*. Transporters such as LacY and MelB are inhibited by the dephosphorylated form of IIA^{Glc}, which is homologous to the LacS-IIA domain. Since structural changes upon phosphorylation of IIA^{Glc} are limited to small shifts (<1.5 Å) of active site residues (*39*) and the interaction surfaces of IIA^{Glc} with HPr, IIB^{Glc}, glycerol kinase, and LacY largely overlap (*40*−*42*), it seems likely that the LacS carrier domain interacts with the equivalent interface on the LacS-IIA domain. Most likely, specific contact points at the interaction surface on the cytoplasmic face of the carrier domain of LacS and the LacS-IIA domain govern the opposite mode of regulation.

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