Functional Interactions between the Subunits of the Lactose Transporter from *Streptococcus thermophilus*

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Although the quaternary state has been assessed in detail for only a few members of the major facilitator superfamily (MFS), it is clear that multiple oligomeric states are represented within the MFS. One of its members, the lactose transporter LacS from *Streptococcus thermophilus* assumes a dimeric structure in the membrane and *in vitro* analysis showed functional interactions between both subunits when proton motive force (Δp)-driven transport was assayed. To study the interactions in further detail, a covalent dimer was constructed consisting of in tandem fused LacS subunits. These covalent dimers, composed of active and completely inactive subunits, were expressed in *Escherichia coli*, and initial rates of Δp-driven lactose uptake and lactose counterflow were determined. We now show that also *in vivo*, both subunits interact functionally; that is, partial complementation of the inactive subunit was observed for both transport modes. Thus, both subunits interact functionally in Δp-driven uptake and in counterflow transport. In addition, analysis of in tandem fused LacS subunits containing one regulatory LacS-IIA domain showed that regulation is primarily an intramolecular event.

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### Introduction

The major facilitator superfamily (MFS) forms the largest family of secondary transporters. Its members, found in all living organisms, catalyze the translocation of a variety of substrates among which are sugars, peptides, anions, cations and hydrophobic compounds. Most MFS proteins consist of 12 α-helical transmembrane-spanning segments (TMS) that are connected by loop regions ranging from a few amino acid residues to several dozens of residues. Although data on functional and structural aspects of these proteins are produced at a great pace, information on the quaternary state(s) is scarce. For both the lactose transporter LacY and the sugar-phosphate antiporter UhpT from *Escherichia coli*, the monomeric unit is sufficient for transport, and earlier claims for LacY dimers are thought to be based on improper experimentation and inaccurate data interpretation. For the anion-exchanger AE1 and glucose transporter GLUT1 from human, and the tetracycline transporter TetA from *E. coli*, on the other hand, there is compelling evidence that these transporters form higher oligomeric structures, and functional interactions between the subunits of the oligomers have been shown.

For secondary transporters outside the MFS, oligomeric states higher than the monomer have been proposed (e.g. the Na+/H+ antiporter NhaA and the multidrug exporter AcrB from *E. coli*, the Na+/glycine betaine symporter BetP from *Corynebacterium glutamicum*, and the human glial glutamate transporter hEAAT2). For most of these transporters, it remains to be determined whether functional interactions between subunits take place. Furthermore, in those cases where cooperativity between subunits has been shown, the role of the oligomeric structure in the mechanism of transport is still far from clear.

Members of the galactoside-pentoside-hexuronide transporter family (GPH family) of the MFS are of pro- or eukaryotic origin and catalyze the transport of sugars and sugar
derivatives in symport with cations. These transporters consist of 12 $\alpha$-helical transmembrane segments that span the membrane in a zig-zag fashion. Detailed analysis of the oligomeric state has been conducted only for the lactose transporter LacS from \textit{Streptococcus thermophilus}, and this system forms a structural and functional dimer. For the xyloside transporter XylP from \textit{Lactobacillus pentosus}, a dimeric structure has been reported. On the other hand, the projection structure of another GPH family member, the melibiose permease MelB from \textit{E. coli}, does not comply with a dimeric state.

Both XylP and LacS have been analyzed in the detergent-solubilized state using blue native gel electrophoresis and analytical ultracentrifugation, and shown to be in a dynamic monomer to dimer equilibrium. The extent of dimerization could be manipulated by varying the detergent type or concentration. The membrane-embedded oligomeric state of XylP and LacS has been determined using freeze-fracture electron microscopy, which suggested that both proteins are present in the bilayer as dimers only. Additional evidence for this dimeric state of LacS in the membrane came from saturation-transfer electron spin resonance studies. Chemical cross-linking of cysteine substitution mutants of LacS \textit{in situ} suggested that on the extracellular side of LacS, TMS V and VIII, and on the cytoplasmic side TMS VI and VII, are located near the centre of the LacS dimer. By studying LacS heterodimers of active (LacS(C320A)) and (conditionally) inactive (LacS(E67C/C320A)) species in proteoliposomes, it has been shown that the subunits within the dimer cooperate. Cooperativity was observed during lactose accumulation driven by the proton motive force ($\Delta p$), but not for the lactose exchange mode of transport.

Within a LacS subunit, two domains can be discerned. The N-terminal membrane-embedded carrier domain catalyzes the translocation event, The C-terminal hydrophilic LacS-IIA domain, unique for the LacS subfamily within the GPH family, is homologous to IIAGlc domains of the PEP phosphotransferase system and resides at the cytoplasmic face of the membrane. The LacS-IIA domain is not essential for transport, but serves a regulatory role. Phosphorylation of LacS-IIA by HPr(His\_P) enables the domain to interact with the carrier domain and modulate the transport activity (our unpublished results).

In order to increase our insights into the functional role(s) of the subunit interactions, heterodimers were formed by fusing two different LacS carrier domains in tandem. In whole \textit{E. coli} cells, functional interactions between a LacS(D71C/C320A) subunit, which is inactive in all modes of transport but still adopts a conformation capable of substrate-binding, and the active LacS(C320A) subunit were analyzed. In addition, the regulation of the LacS carrier domain by LacS-IIA was studied in an asymmetric covalent dimer, in which only one subunit was equipped with a LacS-IIA domain.

**Results**

A covalent dimer to study subunit interactions

Functional interactions between subunits in...
dimeric proteins have been studied by mixing active and inactive species in different ratios and characterizing the resulting heterodimers which constitute at most 50% of all dimers; that is, at an equal ratio of both species, as shown in Figure 1(a) (continuous line). If the association is random, each of the homodimers makes up 25% of all dimers at an equal ratio of both species. If two species do not interact or function independently, the total activity is determined only by the percentage of active species and will decrease linearly (Figure 1(b), dotted line). However, if both species do interact functionally and the phenotype of one of the species dominates the activity of the heterodimer, the activity will follow a quadratic relationship, as shown in Figure 1(b) (continuous lines); lines a and b are obtained when a subunit has a negative- or positive-dominant effect on the opposing subunit, respectively.

To determine if the subunits function in a cooperative manner, the specific activity of the heterodimer needs to be resolved. When the exact ratio of the two species is known (e.g. in a proteoliposomal system) this can be determined from the summed activity of all species. However, in whole cells, the ratio of two separately expressed subunits is difficult to control, because of variations in the expression and inaccuracies in the determination of protein levels from, for instance, immunoblots. By covalent coupling of subunits, the ratio is known beforehand, which has the additional advantage that the maximum percentage of heterodimers can be elevated from 50% to 100% (see Figure 1(a)). This increases the signal that discriminates between independent functioning and negative or positive-dominant effects of subunits, as shown in Figure 1(b).

In order to control the total amount of protein in a reproducible way, expression of the covalent dimer in E. coli MC1061 was governed by the arabinose-inducible $p_{BAD}$ promoter, which proved to be a more convenient and reliable expression system than the vector wherein LacS expression was controlled by its endogenous promoter (our unpublished results).

Construction and functional expression of a covalent LacSΔIIA$_2$ dimer

The minimal unit of LacS able to catalyze substrate translocation is the membrane-embedded carrier domain (our unpublished results). To study the functional interactions of the carrier domains in a defined manner, the covalent LacS dimer comprised two joined LacS-IIA subunits rather than two LacS subunits. LacS-IIA lacks the C-terminal LacS-IIA domain that can be phosphorylated by HPr(His$\gamma$-P) (our unpublished results), but shows equal rates of $\Delta p$-driven lactose transport and lactose counterflow as unphosphorylated full-length LacS (our unpublished results).

Twenty-eight amino acid residues follow the predicted end of TMS 12 of the first LacS-IIA(C320A) subunit. An artificial sequence of 15 amino acid residues was used to link the two subunits. At the DNA level, the linker region contains four endonuclease restriction sites and a sequence coding for a TEV protease recognition site, yielding the protein sequence GSGDQENLYFQGTSA. Together with the 18 amino acid residues preceding the predicted start of the first TMS of the second LacS-IIA(C320A) subunit, the total linker region connecting both subunits comprises 61 amino acid residues (Figure 2). This covalent dimer, in which both subunits contain the C320A mutation, was designated LacSΔIIA$_2$(CC).

As shown in Figure 3, LacSΔIIA$_2$(CC) catalyzed $\Delta p$-driven lactose uptake in whole E. coli MC1061 cells, demonstrating its functional expression and membrane insertion. Maximal transport activity of LacSΔIIA$_2$ was observed when cells were induced with $2 \times 10^{-3}$% (w/v) L-arabinose, whereas induction with $1 \times 10^{-3}$% (w/v) L-arabinose yielded maximal transport activity of LacSΔIIA (results not shown). At these optimal concentrations of inducer, the initial rates of $\Delta p$-driven lactose uptake for LacSΔIIA$_2$(CC) and LacSΔIIA(C320A) were 2.6 nmol of lactose/mg of protein per minute and 11 nmol of lactose/mg of protein per minute, respectively. Analysis of the levels of LacSΔIIA$_2$.

**Figure 2.** Topology model of the LacSΔIIA$_2$ dimer. Membrane topology of the LacS-IIA subunits is based on the model of MelB. The gray horizontal lines indicate the membrane interfaces. Asp71 in the second transmembrane segment (TMS) of each subunit is depicted in black. The LacS-IIA subunits are coupled via a linker of 61 amino acid residues (gray circles). The sequence of the artificially introduced stretch of 15 residues in the linker is shown. In all subunits, the endogenous Cys320 in TMS IX (also shown in black) was replaced by an alanine residue.
Inactive D71C/C320A subunits are complemented by active C320A subunits within LacS\textsubscript{DIIA2} during Δp-driven and counterflow transport

Heterodimeric LacS\textsubscript{DIIA2} derivatives were constructed, comprising the D71C mutation in either the first or the second subunit and using the C320A background. The D71C mutation renders LacS inactive in all modes of transport, but does not affect the overall structure of the transporter, as the capacity of LacS(D71C/C320A) to bind substrate was retained.\textsuperscript{24} The LacS\textsubscript{DIIA2} derivatives containing the D71C/C320A mutation in the first, last, or both subunits were designated LacS\textsubscript{DIIA2}(DC), LacS\textsubscript{DIIA2}(CD), and LacS\textsubscript{DIIA2}(DD), respectively. The mutant variants of LacS\textsubscript{DIIA2} were expressed to comparable levels (Figure 4(a)), enabling a direct comparison of the transport activities.

Like the strain containing a control plasmid, cells expressing LacS\textsubscript{DIIA2}(DD) showed no significant uptake of lactose in whole \textit{E. coli} MC1061 cells (Figure 3). The transport rates of LacS\textsubscript{DIIA2}(CD) and LacS\textsubscript{DIIA2}(DC) were equal, indicating that both halves of the forced dimer are correctly inserted in the membrane. The observed rates of Δp-driven lactose uptake of LacS\textsubscript{DIIA2}(CD) and LacS\textsubscript{DIIA2}(DC) were approximately 80% of the initial rate of uptake of LacS\textsubscript{DIIA2}(CC) (Figures 3 and 5). This result suggests that the D71C/C320A subunit is partially complemented by the active C320A subunit.

Figure 3. Δp-driven lactose transport by LacS\textsubscript{DIIA2} derivatives in \textit{E. coli} MC1061. The accumulation of lactose driven by the proton motive force was measured in duplicate for LacS\textsubscript{DIIA2}(CC) (triangles pointing downwards), LacS\textsubscript{DIIA2}(CD) (squares), LacS\textsubscript{DIIA2}(DC) (diamonds), LacS\textsubscript{DIIA2}(DD) (triangles pointing upwards), and MC1061 cells containing an empty plasmid with an ampicillin-resistance marker (pBluescript IIISK+ (Stratagene)) (circles).

Figure 4. Immunoblots of covalently linked LacS derivatives, which were detected with an antibody directed against a hexa-His-tag. (a) Immunoblots of LacS\textsubscript{DIIA2} derivatives, purified from equal amounts of cells. CC and DD represent homodimers of C320A or D71C/C320A subunits, respectively. CD and DC represent heterodimers with a D71C/C320A subunit in the second or first subunit, respectively. (b) Immunoblots of the LacS\textsubscript{DIIA2}-LacS derivatives, purified from equal amounts of cells. The same notation as that used in (a) indicates the different LacS\textsubscript{DIIA2}-LacS derivatives.

Lactose counterflow transport by the LacS\textsubscript{DIIA2} derivatives showed a profile similar to that observed for Δp-driven lactose uptake (Figure 5). LacS\textsubscript{DIIA2}(DD) was completely defective in transport, and both

Figure 5. Initial rates of Δp-driven lactose uptake and lactose counterflow by LacS\textsubscript{DIIA2} derivatives in \textit{E. coli} MC1061. Counterflow and Δp-driven lactose transport rates were measured on cell suspensions derived from the same culture. To measure lactose counterflow transport, de-energized cell suspensions were preloaded with 10 mM lactose and diluted into buffer containing 100 \(\mu\text{M} [\text{14C}]\)lactose. Cells used to measure lactose transport driven by the Δp were pre-energized for two minutes by incubation with 10 mM D-Li-lactate and aeration. Subsequently, lactose accumulation was started by the addition of 50 \(\mu\text{M} [\text{14C}]\)lactose. Each rate reflects the average from two independent experiments. Open and hatched bars reflect initial lactose uptake rates for Δp-driven and lactose counterflow transport, respectively.
LacSΔIIA2(DD) and LacSΔIIA2(DC) showed approximately 80% of the initial rate of lactose counterflow by LacSΔIIA2(CC), indicating that for counterflow transport also the D71C/C320A subunit is partially complemented by the active C320A subunit.

**Covalent fusion of LacSΔIIA subunits differently affects counterflow and Δp-driven lactose transport**

Under identical experimental conditions, non-covalently linked LacS(C320A) showed approximately half the rate of Δp-driven transport compared to lactose counterflow (9.0 nmol of lactose/mg of protein per minute and 20 nmol of lactose/mg of protein per minute, respectively), while LacSΔIIA(C320A) showed comparable rates for both modes of transport (11 nmol of lactose/mg of protein per minute and 8.6 nmol of lactose/mg of protein per minute). This is most probably caused by a stimulation of the counterflow reaction of LacS(C320A) by HPr(His\(\sim\)P) mediated phosphorylation of LacS-IIA (our unpublished results).

Whereas the ratio of the initial rates of Δp-driven and counterflow was 0.5 and 1.3 for LacS and LacSΔIIA, respectively, this ratio was approximately 3 for the CC, CD and DC derivatives of LacSΔIIA2 (summarized in Table 1). Since the initial rates of both Δp-driven and counterflow lactose transport were determined on cells derived from the same culture, the discrepancy in relative activities cannot be caused by variation in expression levels. Effects of variations in internal pH between both transport modes on LacSΔIIA2 derivatives could be excluded, because the pH of the buffer during lactose counterflow transport was adjusted to 7.7, which was equal to the internal pH of cells during Δp-driven transport. Furthermore, by charging the cells with \(^{14}\text{C}\)lactose, it was shown that the MC1061 cells containing LacSΔIIA2 were equilibrated with lactose to levels similar to that of cells containing non-covalently linked LacSΔIIA(C320A), ruling out limiting intracellular lactose concentrations as a cause for the decreased counterflow transport rates. We, therefore, conclude that the threefold difference in initial rates of Δp-driven lactose transport and lactose counterflow transport by the CC, CD, and DC derivatives of LacSΔIIA2 is a genuine property of these dimers.

A more detailed kinetic analysis of lactose exchange transport, which comprises the same kinetics steps as counterflow transport and differs from counterflow only in substrate concentrations and the location of the \(^{14}\text{C}\) isotope of lactose (inside for exchange, outside for counterflow), was conducted (Figure 6). All LacSΔIIA2 derivatives showed similar lactose exchange kinetics, again reflecting that all subunits (irrespective of the order of active C320A and inactive D71C/C320A subunits) were affected equally by the covalent linkage. Moreover, the apparent affinity constants of the LacSΔIIA2 derivatives for lactose were maximally increased only twofold compared to the \(K_{\text{app}}^{\text{p}}\) of LacS-IIA, suggesting that the overall structure of both subunits in the LacSΔIIA2 derivatives is conserved. Summarizing, it seems that both subunits within the LacSΔIIA2 derivatives adopt a correct conformation.

**Table 1. Overview of the topology and transport rates of the LacS derivatives**

| LacS derivative       | Topology | Initial transport rate (nmol lactose/mg protein per min)\(a\) | Ratio Δp-driven/counterflow |
|----------------------|----------|-------------------------------------------------------------|----------------------------|
| LacS(C320A)          |          | 9.04 ± 1.77                                                 | 0.45                       |
| LacSΔIIA(C320A)      |          | 11.14 ± 0.59                                                | 1.30                       |
| LacSΔIIA2(CC)        |          | 2.57 ± 0.21 (100)                                           | 3.17                       |
| LacSΔIIA2(CD)        |          | 2.10 ± 0.08 (82)                                            | 3.33                       |
| LacSΔIIA2(DC)        |          | 2.04 ± 0.25 (79)                                            | 3.34                       |
| LacSΔIIA2(DD)        |          | 0.01 ± 0.03 (0)                                             | X                          |
| LacSIIA-LacS(CC)     |          | 2.83 ± 0.35                                                 | 0.63                       |
| LacSΔIIA-LacS(CD)    |          | 2.92 ± 0.29                                                 | 1.69                       |
| LacSΔIIA-LacS(DC)    |          | 0.59 ± 0.21                                                 | 0.63                       |
| LacSΔIIA-LacS(DD)    |          | 0.07 ± 0.08                                                 | X                          |

The LacSΔIIA C320A and D71C/C320A subunit are presented as a rectangle, and a rectangle filled with a cross, respectively. The LacS-IIA domain is depicted as a flattened sphere. The initial transport rates were determined as described in the legend to Figure 5.

\(a\) Values within parentheses are percentages.
The LacS-IIA domain interacts primarily with the subunit of LacS\_IIA-LacS to which it is attached

Upon HP\(r\)(His \~{} P)-mediated phosphorylation of His552 in the LacS-IIA domain, the LacS-IIA domain interacts with the carrier and thereby stimulates lactose counterflow (our unpublished results).\(^\text{23}\) To determine whether the regulation of the LacS-IIA domain occurs inter- or intramolecularly, a tandem fusion was constructed in which the last subunit has a C-terminal LacS-IIA domain attached to the carrier domain, creating a topology equal to the full-length LacS protein. This construct is designated LacS\_IIA-LacS. The mutant variants derived from this construct, harboring the D71C mutation, were not expressed to an equal level, as shown in Figure 4(b). Constructs containing the D71C substitution in the second subunit were expressed to a higher level than subunits without a D71C mutation. This variation in expression levels allowed only a qualitative analysis, since exact quantification of the amount of functional transporters was not possible at this stage, due to the lack of a suitable ligand-binding assay.

The rate of \(\Delta p\)-driven lactose uptake of LacS\_IIA-LacS was in the same range as the rate of LacS\_IIA\_D\(\text{CC}\) (2.8 nmol of lactose/mg protein per minute and 2.6 nmol of lactose/mg protein per minute, respectively) (Figure 7). Furthermore, the kinetics of lactose exchange by LacS\_IIA\_LacS(CC) yielded an apparent affinity constant for lactose similar to that of the other LacS derivatives (Figure 6). In line with the observations on the LacS\_IIA\_ derivatives, both LacS\_IIA\_LacS(CC) and LacS\_IIA\_LacS(DC) were active in \(\Delta p\)-driven transport, whereas LacS\_IIA\_LacS(DD) was inactive (Figure 7).

As described above, the ratio of the initial rates of \(\Delta p\)-driven transport over counterflow was 0.5 and 1.3 for LacS and LacS\_IIA, respectively, while this ratio was close to 3 for the CC, CD and DC derivatives of LacS\_IIA\(\text{CC}\) (summarized in Table 1). Both LacS\_IIA\_LacS derivatives that had a LacS-IIA domain attached to the active C320A subunit (LacS\_IIA\_LacS(CC) and LacS\_IIA\_LacS(DC)) showed a ratio of \(\Delta p\)-driven transport over counterflow of approximately 0.6. In contrast, this ratio was elevated to 1.7 for LacS\_IIA\_LacS(DD). Since the ratio differs for the LacS\_IIA\_LacS(DD) and LacS\_IIA\_LacS(DD) constructs, this suggests strongly that the IIA domain is restricted in its opportunity to interact with the carrier domains within the LacS dimer. Most likely, it cannot interact with any nearby subunit but only with the subunit to which it is attached.

Discussion

Several independent studies led to the conclusion that the detergent-solubilized LacS protein is in monomer to dimer equilibrium,\(^\text{8,25,20}\) whereas...
membrane-embedded LacS is dimeric. Veenhoff et al. showed by in vitro analysis of membrane-reconstituted (conditionally) inactive LacS(E67C/C320A) and active LacS(C320A) species that, within this structural dimer, functional interactions between the subunits take place in Δp-driven uptake but not in counterflow transport. Here, we report the first in vivo demonstration of functional interactions between the subunits within the LacS dimer and show that both subunits functionally interact in Δp-driven transport and in counterflow. We show that: (i) the activity of the D71C mutant is (partially) restored by an active subunit; (ii) covalent linkage of two LacS subunits increases the rate of Δp-driven uptake relative to counterflow transport; and (iii) phosphorylation of the LacS-IIA domain stimulates the trans subunit of dimeric LacS.

Due to its uncoupled phenotype in whole cells, resulting in rapid efflux of lactose down the concentration gradient, LacS(E67C/C320A), previously used to demonstrate functional interactions within the LacS dimer in vitro, could not be used to study subunit interactions in whole cells. Instead, the translocation-defective but lactose binding-competent LacS(D71C/C320A) was employed. Both Glu67 and Asp71 are located in TMS II, and acidic residues at these positions are highly conserved throughout the GPH family. Glu67 has been proposed to be involved in coupling proton and galactoside transport, whereas Asp71 is thought to contribute to the proton binding site. In addition, both residues are in a region that is conformationally active upon substrate-binding. Membrane-reconstituted LacS(E67C/C320A) catalyzes lactose counterflow transport at ~80% of the rate of LacS(C320A) and after modification of Cys71 with NEM ~30% activity remained. LacS(E67C/C320A) is completely defective in Δp-driven lactose transport. In contrast, LacS(D71C/C320A) is inactive in both Δp-driven lactose transport and lactose counterflow, but the overall structure of the protein is retained as substrate-binding still occurs. Several second-site suppressor mutations of Cys71 have been isolated, among which R230C, which could restore lactose counterflow transport but not Δp-driven lactose transport. By employing LacS(D71C/C320A) in tandem constructs with LacS(C320A), the effect of a subunit defective in all transport modes could be determined.

The LacSΔIIA2 derivatives were expressed functionally, although the levels were lower than those of the free subunits, as observed before for other in tandem fusion proteins. The expression levels of the different LacSΔIIA2 derivatives were equal, as judged by immunoblotting (Figure 4(a)), but this technique lacks the sensitivity to demonstrate small variations (<20%) in expression levels. The LacSΔIIA2(CD) was functional in both Δp-driven and lactose counterflow transport, and both subunits are membrane-inserted, as LacSΔIIA2(CD) and LacSΔIIA2(DC) showed similar transport rates. LacSΔIIA2-DD), which resembles the LacS(D71C/C320A) homodimer, is inactive, indicating that the mere coupling of the subunits is not sufficient for the restoration of the transport capacity. The difference in the ratio between the initial rates of Δp-driven lactose transport and lactose counterflow for the LacSΔIIA2 derivatives when compared with LacSΔIIA and LacS, suggest that either Δp-driven lactose transport benefits or lactose counterflow activity deficits from the fusion of the subunits (Table 1). Kinetic analysis of lactose exchange transport showed that substrate-binding of the LacSΔIIA2 derivatives was affected only slightly, because the $K_{\text{app}}$ was at most twofold higher than the $K_{\text{app}}$ of the LacSΔIIA dimer. Taken together, the covalent coupling of subunits introduces only some moderate changes within the LacS dimer and seems a valid system to study subunit interactions.

For independent operating subunits within the LacS dimer, the heterodimers LacSΔIIA2(CD) and LacSΔIIA2(DC) would be expected to show half the initial transport rates of LacSΔIIA2(CC). A negative dominant phenotype of the D71C subunit would yield inactive LacSΔIIA2(CD) and LacSΔIIA2(DC) heterodimers. Clearly, both scenarios do not apply, since the initial rates of LacSΔIIA2(CD) and LacSΔIIA2(DC) for both Δp-driven lactose transport and lactose counterflow were approximately 80% of the rates of LacSΔIIA2(CC) (Table 1). Rather, these transport activities support the contention that the C320A subunit has a positive-dominant effect, since it is able to partly restore the activity of the LacSΔIIA(D71C/C320A) subunit. Our new findings differ in some aspects from previous observations, using a proteoliposomal system, which demonstrated functional interactions between the LacS subunits for Δp-driven lactose transport only. In proteoliposomes, increasing the percentage of impaired LacS(E67C/C320A) over LacS(C320A) resulted in a linear decrease in lactose counterflow transport, suggesting that the subunits function independently during this mode of transport. Furthermore, heterodimers composed of inactive LacS(E67C/C320A) and active LacS(C320A) were shown to be completely inactive in Δp-driven lactose transport, indicating negative dominance of the LacS(E67C/C320A) subunit. In the present study, negative dominance of the LacSΔIIA(D71C/C320A) subunit in LacSΔIIA2(CD) or (DC), is clearly not observed. Rather than interpreting these discrepancies in terms of differences in the experimental context (proteoliposomes versus intact cells), we feel that they can be explained by the different phenotypes of the LacS(E67C/C320A) and LacS (D71C/C320A) mutants. Most likely, LacS(D71C/C320A) is locked in one conformation, since it has lost its ability to reorient its substrate-binding sites as required for transport while retaining the ability to bind substrate. An opposing functional subunit could enable the LacSΔIIA(D71C/C320A) to overcome this locked conformation, leading to (partial) complementation. LacS(E67C/C320A), on the other hand, kept the ability to reorient its binding sites.
and thereby catalyze lactose counterflow transport. Only its ability to catalyze Δp-driven lactose transport was impaired. As the extent of the impairment is different for both mutants, this may explain the lack of complementation of LacS(E67C/C320A) by LacS(C320A).

Apart from the relative activities of the different LacSΔIII_A2 derivatives for either Δp-driven lactose uptake or lactose counterflow, the ratio of the initial rates of Δp-driven lactose transport over counterflow reveals interesting features. This ratio is approximately 3 for the CC, CD and DC derivatives of LacSΔIII_A2 while it is close to 1 for LacSΔIIIA. It is likely that the difference in the ratio of the initial rates for LacSΔIII_A2 compared to LacSΔIIIA is a specific property of the fusion protein, caused by the presence of the linker connecting both subunits. A possible consequence of the covalent coupling could be the decreased ability of the subunits to separate transiently.

Additionally, the mere presence of the LacS-IIA domain to the second subunit decreased the ratio of Δp-driven transport over counterflow from ~3 to 0.6–1.7, indicating that phosphorylation of this domain affects the subunit interactions. Since the absolute rates of Δp-driven lactose transport of LacSΔIII_A2(CC) and LacSΔIIIA-LacS(CC) are similar, it seems that the rate of lactose counterflow transport is increased upon addition of the LacS-IIA domain. Therefore, it is tempting to speculate that the kinetic step that is impaired by the coupling of the subunits is the same as the one that is stimulated by the phosphorylation of the LacS-IIA domain. Thus, the phosphorylated LacS-IIA domain could induce a transient conformation of the carrier domain that is less strongly interacting with the opposing subunit.

Under the conditions used, the IIA-domain of LacS is phosphorylated in _E. coli_ MC1061, resulting in an increase in the rate of counterflow transport and a decrease in the ratio of Δp-driven transport over counterflow (our unpublished results). Phosphorylation of the LacS-IIA domain in the LacSΔIII_A2-LacS derivatives resulted in a ratio of Δp-driven transport over counterflow near 0.6 if the LacS-IIA domain was associated with the LacSΔIIIA(C320A) subunit (Figure 7), and near 1.7 if it was associated with the inactive D71C subunit. The difference in the ratio of both transport modes observed for LacSΔIII_A2-LacS(CD) and LacSΔIIIA-LacS(CD) suggests that the LacS-IIA domain prefers to functionally interact with the subunit to which it is attached. Although the rate of lactose counterflow is increased relatively for LacSΔIIIA-LacS(CD), the increase is smaller than that observed for the CC and DC derivatives of LacSΔIIIA-LacS, indicating that the D71C/C320A subunit cannot be stimulated by phosphorylated LacS-IIA to the same extent as the C320A subunit.

Taken together, the data presented here strengthen the conclusion that the two subunits of the LacS dimer interact functionally, as separate proteins in vitro and in tandem fusions _in vivo_. The functional interactions within the LacS dimer take place during both Δp-driven lactose transport and lactose counterflow, and can be both positive and negative of nature. Furthermore, the LacS-IIA domain primarily stimulates transport through intramolecular interactions with the carrier domain.

### Materials and Methods

#### Bacterial strain

_E. coli_ JM110[^30] was used for intermediate cloning steps. The final constructs were expressed in _E. coli_ MC1061 (relevant genotype: ΔlacZY, araBAD[^31] ). Both strains were cultivated at 37°C on Luria broth under vigorous aeration. When appropriate, the medium was supplemented with 50 μg/ml of ampicillin.

#### Plasmid constructions

DNA manipulations were done according to standard protocols. The construction of pBADlacSC320A, pSKE8EhisC320A-BamH1-IIA, pNlacSC320Ahis and pSKlacSC320AIIA will be described elsewhere. Plasmids pSKE8EhisC320A and pSKE8EhisC320A/D71C have been described.[^24]

**pSKlacSC320A/D71C/IIA**

The Ncol-Xhol fragment of pSKlacSC320AIIA was replaced by the 2147 bp Ncol-Xhol fragment from pSKE8EhisC320A/D71C.

**pBADlacSIIIAlacSIII/IIA**

In order to incorporate the D71C mutation in the first subunit, the 200 bp AatII-Ncol fragment from pSKE8EhisC320A-BamH1-IIA was replaced by the AatII-Ncol fragment from pSKE8EhisC320A/D71C, yielding pSKE8EhisC320A/D71C-BamH1-IIA. Plasmid pSKE8EhisC320A/D71C-BamH1-IIA was digested with BamH1-SpeI and ligated to a linker of two annealed oligonucleotides (linkerBS and linkerSR; see Table 2) with extensions resembling a BamH1 or an SpeI overhang. The AatII-XbaI fragment of this product was ligated into AatII-XbaI-digested pBADlacSC320A, yielding pBADsub1C320A/D71C.

In order to construct the plasmid containing the second subunit, pNlacSC320Ahis was digested with BclI-Ncol and the 389 bp fragment was replaced by a linker of two annealed oligonucleotides (linkerBN and linkerNR; see Table 2) with extensions resembling a BclI or an Ncol overhang, yielding pBADsub2C320A+IIA. The sequence coding for the LacS-IIA-domain was removed by exchange of the 2229 bp AatII-XbaI fragment for the 1723 bp AatII-XbaI fragment from pSKlacSC320A/D71C/IIA, producing pBADsub2C320A/A/D71C/IIA.

To link both subunits, the 34 bp BclI-XbaI fragment from pBADsub1C320A/D71C was exchanged for the 2333 bp BclI-XbaI fragment from pBADsub2C320A/A/D71C/IIA containing the second subunit, yielding pBADlacSIIIAlacSIII/IIA. Four derivatives of pBADlacSIIIAlacSIII/IIA were generated, containing the D71C mutation in the first, the last, or both subunits.
Table 2. The complementary oligonucleotides constituting parts of the linker connecting both LacS-carrier domains

| Primer    | Sequence                                                                 |
|-----------|--------------------------------------------------------------------------|
| LinkerBS  | 5′gatccgggtgatcaggagaacccctatttttcaaggca                                  |
| LinkerSB  | 5′ctagctttgctaataggtgctcttcgatcaccgg                                   |
| LinkerBN  | 5′gatcggagaacctctattttttcaaggca                                       |
| LinkerNB  | 5′ctagcgactagtgcctaataggtgctcttccttct                                   |

LinkerBS and LinkerSB constitute a double-stranded linker with artificial BamHI and SpeI overhangs. LinkerBN and LinkerNB constitute a double-stranded linker with artificial BclI and NcoI overhangs. The sequence of both double-stranded linkers partially overlaps; double-stranded linkers were created by mixing both oligonucleotides in equal ratios, boiling for five minutes and annealing by cooling slowly to room temperature.

pBADlacS-ΔIIAlacS + ΔIIA

The construction of the vector harboring a gene coding for a fusion between a LacS carrier domain and full-length LacS was similar to the construction of the vector for two fused LacS carrier domains. Instead of pBAD-sub2C320A(/ΔD71C)IIA, plasmid pBADsub2C320A(/ΔD71C) + II A was used. The ΔD71C mutation was added by exchanging the 2229 bp AatII-XbaI fragment of pBADlacS-ΔIIAlacS + ΔII A for the 2229 bp AatII-XbaI fragment of pSKE8EhisC320A/D71C.

Whole cell transport assays

Cultivation

E. coli MC1061 cells were cultivated, washed and concentrated as will be described elsewhere. Cultures were induced with 1×10^{-5} % and 2×10^{-5} % (w/v) L-arabinose when LacS derivatives or LacS-LacS fusion proteins were expressed, respectively. Cells used for lactose exchange transport were induced with 1.5×10^{-4} % (w/v) L-arabinose when LacS derivatives or LacS-LacS fusion proteins were deprived of DTT by washing and subsequently solubilized as described.

Transport assays

General handlings involved in lactose transport in E. coli MC1061 cells and the preparation of cells to be used for Δp-driven lactose uptake and lactose counterflow transport will be described elsewhere.

Lactose exchange transport

Cells were concentrated to 45 mg protein/ml and incubated overnight in KPM (50 mM KPi (pH 7.7), 2 mM MgSO4) plus 5 mM [14C]lactose. The next day, cells were de-energized by incubation with the protonophore SF6847 (50 μM) plus 30 mM NaN3 for two hours. Lactose exchange assay was assayed at 20 °C by 100-fold dilution of the cells into KPM, supplemented with 50 μM SF6847. The external lactose concentration varied from 50 μM to 20 mM.

Membrane vesicle isolation

Inside-out membrane vesicles from E. coli MC1061 cells were prepared as described. Membrane vesicles were resuspended in 50 mM KPi (pH 7), plus 3 mM DTT, frozen in liquid nitrogen and stored at −80 °C. The protein concentration was determined using the DC protein assay (Bio-Rad).

Purification and immunodetection of LacS–LacS fusion proteins

All steps during the purification were performed at 4 °C. E. coli MC1061 membrane vesicles (approximately 6 mg of total protein) containing LacS fusion proteins were deprived of DTT by washing and subsequently solubilized as described.

Samples were analysed by SDS-PAGE, semi-dry electrophoretic transfer and subsequent immunodetection with a primary antibody directed against a hexa-His-tag (Amersham Pharmacia Biotech) as described.

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