

# Unidirectional Reconstitution into Detergent-destabilized Liposomes of the Purified Lactose Transport System of *Streptococcus thermophilus*\*

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The lactose transport protein (LacS) of *Streptococcus thermophilus* was amplified to levels as high as 8 and 30% of total membrane protein in *Escherichia coli* and *S. thermophilus*, respectively. In both organisms the protein was functional and the expression levels were highest with the streptococcal *lacS* promoter. Also a LacS deletion mutant, lacking the carboxyl-terminal regulatory domain, could be amplified to levels >20% of membrane protein. Membranes from *S. thermophilus* proved to be superior in terms of efficient solubilization and ease and extent of purification of LacS; >95% of LacS was solubilized with relatively low concentrations of Triton X-100, *n*-octyl- $\beta$ -D-glucoside, *n*-dodecyl- $\beta$ -D-maltoside, or C<sub>12</sub>E<sub>8</sub>. The LacS protein carrying a poly-histidine tag was purified in large quantities (~5 mg/liter of culture) and with a purity >98% in a two-step process involving nickel chelate affinity and anion exchange chromatography. The membrane reconstitution of LacS was studied systematically by stepwise solubilization of preformed liposomes, prepared from *E. coli* phospholipid and phosphatidylcholine, and protein incorporation at the different stages of liposome solubilization. The detergents were removed by adsorption onto polystyrene beads and H<sup>+</sup>-lactose symport and lactose counterflow were measured. Highest transport activities were obtained when Triton X-100 was used throughout the solubilization/purification procedure, whereas activity was lost irreversibly with *n*-octyl- $\beta$ -D-glucoside. For reconstitutions mediated by *n*-dodecyl- $\beta$ -D-maltoside, C<sub>12</sub>E<sub>8</sub>, and to a lesser extent Triton X-100, the highest transport activities were obtained when the liposomes were titrated with low amounts of detergent (onset of liposome solubilization). Importantly, under these conditions proteoliposomes were obtained in which LacS was reconstituted in an inside-out orientation, as suggested by the outside labeling of a single cysteine mutant with a membrane impermeable biotin-maleimide. The results are consistent with a mechanism of reconstitution in which the hydrophilic regions of LacS prevent a random insertion of the protein into the membrane. Consistent with the *in vivo* lactose/galactose

exchange catalyzed by the LacS protein, the maximal rate of lactose counterflow was almost 2 orders of magnitude higher than that of H<sup>+</sup>-lactose symport.

The lactose transport protein (LacS) of *Streptococcus thermophilus* is a hybrid protein composed of a polytopic membrane domain that is predicted to span the cytoplasmic membrane 12 times and a carboxyl-terminal cytoplasmic domain of about 180 amino acids (Poolman *et al.*, 1989). The hydrophilic domain is homologous to IIA protein(s) (domains) of various phosphoenolpyruvate:sugar phosphotransferase systems and is involved in the regulation of this secondary transport system (Poolman *et al.*, 1995b). The hydrophobic carrier domain, which is sufficient for transport activity, is homologous to a new family of secondary transporters to which belong the melibiose transport proteins (MelB) of *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*, the glucuronide transport protein (GusB) of *E. coli*, the xylose transport protein (XylP) of *Lactobacillus pentosus*, and various other proteins that have not been studied in great detail (Poolman and Konings, 1993; Poolman *et al.*, 1996).

The LacS protein of *S. thermophilus* has been functionally expressed in *E. coli* and was shown to catalyze the transport of a variety of  $\alpha$ - and  $\beta$ -galactosides. Studies in membrane vesicles from *S. thermophilus* fused with cytochrome *c* oxidase containing liposomes showed that the sugars are accumulated by a H<sup>+</sup>-solute symport mechanism driven by both a membrane potential and a pH gradient. The uptake of lactose (precursor) can also be driven by a coupled exchange reaction with galactose (product) without the net movement of protons, which reflects the prominent transport reaction *in vivo* (Poolman, 1990; Foucaud and Poolman, 1992).

In order to elucidate the structure of the protein and the regulation of its activity it was necessary to purify the protein and to develop methods for efficient and unidirectional reconstitution into artificial membranes. Previously, the expression levels in *E. coli* were too low to identify the LacS protein on Coomassie-stained SDS-PAGE<sup>1</sup> gels. In the present study, different expression systems based on different promoters, ex-

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<sup>1</sup> The abbreviations used are: PAGE, polyacrylamide gel electrophoresis;  $\Delta p$  (or pmf), proton motive force; bp, base pair(s); C<sub>12</sub>E<sub>8</sub>, octaethylene glycol monododecyl ether; CMC, critical micelle concentration; CSPD, disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1]decan}-4-yl)phenyl phosphate; MPB, 3-(*N*-maleimidylpropionyl)-biocytin; NTA, nitrilotriacetic acid; PL, phospholipids; PC, phosphatidylcholine; Pipes, 1,4-piperazinediethanesulfonic acid; TMG, methyl-1-thio- $\beta$ -D-galactopyranoside.

TABLE I  
Plasmids used

Carb<sup>R</sup>, Tet<sup>R</sup>, Cm<sup>R</sup>, and Em<sup>R</sup> indicate resistance to carbenicillin, tetracycline, chloramphenicol, and erythromycin, respectively; *rop*, repressor of primer.

Plasmid	Relevant properties	Source/reference
pSKII(+) pSKE8	Carb <sup>R</sup> , high copy expression vector pSKII(+), carrying <i>lacS</i> of <i>S. thermophilus</i> A147 as 4073-bp <i>EcoRI</i> fragment ( <i>galM<sup>+</sup>, lacS<sup>+</sup></i> )	Stratagene Poolman <i>et al.</i> (1992)
pSKE8E	pSKE8 with <i>EcoRI</i> site 21-bp upstream of the initiation ATG of <i>lacS</i>	This work
pSKE8Mlu	pSKE8 with <i>MluI</i> site on the stop codon of <i>lacS</i>	This work
pSKE8His	pSKE8 with his-tagged <i>lacS</i>	This work
pTAQI	Carb <sup>R</sup> , pBR322, <i>lacI</i> behind penicillinase promoter, medium copy number vector, inducible <i>tac</i> promoter	Genencor Int., San Francisco
pLS1	pTAQI, carrying 1965-bp <i>EcoRI-DraI</i> fragment from pSKE8E ( <i>lacS</i> of <i>S. thermophilus</i> ) in <i>EcoRI-SmaI</i> sites	This work
pTTQ18	Carb <sup>R</sup> , high copy number expression vector, inducible <i>tac</i> promoter	Amersham Int. plc
pLL01	pTTQ18, carrying 1965-bp <i>EcoRI-DraI</i> fragment from pSKE8E ( <i>lacS</i> of <i>S. thermophilus</i> ) in <i>EcoRI-SmaI</i> sites	This work
pMTC15	Carb <sup>R</sup> , pBR322 with <i>galP</i> under control of its own promoter ( <i>rop</i> sequence deleted)	Footnote 2
pSKE8N	pSKE8 with <i>NcoI</i> site on the initiation codon of <i>lacS</i>	This work
pLL11	pMTC15 with <i>galP</i> (1404-bp <i>NcoI-HindIII</i> fragment) replaced by 2225-bp <i>NcoI-EcoRI</i> fragment of pSKE8N	This work
pGK13	Cm <sup>R</sup> , Em <sup>R</sup> , <i>E. coli</i> , <i>S. thermophilus</i> shuttle vector	Kok <i>et al.</i> (1984)
pGKGS8	pGK13, carrying <i>lacS</i> of <i>S. thermophilus</i> as 3824-bp <i>EcoRI-DraI</i> fragment from pSKE8 ligated into the <i>EcoRI-EcoRV</i> sites	Poolman <i>et al.</i> (1995b)
pGKGS8( <i>lacS</i> Δ160)	pGKGS8 with 480-bp deletion (deletion of IIA domain)	Poolman <i>et al.</i> (1995b)
pGKHis	pGKGS8 with his-tagged <i>lacS</i>	This work
pSKE8(C320A)	pSKE8 with cysteine 320 of LacS replaced by alanine	This work
pGKH(C320A)	pGKHis with cysteine 320 replaced by alanine	This work
pGKH(C320A/S384C)	pGKH(C320A) with serine 384 replaced by cysteine	This work

pression hosts, and culture conditions were tested, and expression levels were assessed by transport assays, immunoblotting, and staining of total membrane protein after SDS-PAGE. As a result, new expression systems, based on the *lacS* promoter were developed which could be of general use in amplifying membrane proteins, in particular, when used in combination with the Gram-positive bacterium *S. thermophilus* as host.

The most commonly used method for the reconstitution of transport proteins is based on *n*-octyl- $\beta$ -D-glucopyranoside solubilization of membrane vesicles and removal of the detergent by dilution or dialysis (Baron and Thompson, 1975). In general, phospholipids and compounds like glycerol and compatible solutes seem to stabilize the proteins while in the solubilized state (Newman and Wilson, 1980; Chen and Wilson, 1984; Maloney and Ambudkar, 1989; Poolman and Konings, 1993). Although LacS has successfully been reconstituted from octyl glucoside-solubilized membrane extracts, the purified protein was irreversibly inactivated by octyl glucoside. In developing new reconstitution strategies, several detergents and methods of membrane incorporation were tested. It proved that reconstitution based on insertion of the purified LacS protein into preformed, detergent-destabilized liposomes was most successful and highly reproducible.

#### EXPERIMENTAL PROCEDURES

##### Materials

D-[glucose-1-<sup>14</sup>C]Lactose (2.11 TBq/mol) was obtained from the Radiochemical Centre, Amersham, United Kingdom. [<sup>14</sup>C]Methyl- $\beta$ -D-thiogalactopyranoside (1.85 TBq/mol) was obtained from DuPont NEN. Ni-NTA resin was from Qiagen, Inc., the anion exchange column (HR5/5 Mono Q) from Pharmacia Biotech Inc., and Bio-Beads SM-2 from Bio-Rad. Streptavidin-alkaline phosphatase conjugate, *n*-octyl- $\beta$ -D-glucoside, and Triton X-100 were from Boehringer Mannheim; *n*-dodecyl- $\beta$ -D-maltoside and sodium cholate from Sigma; and C<sub>12</sub>E<sub>8</sub> from Fluka Chemie AG. Total *E. coli* lipids and L- $\alpha$ -phosphatidylcholine from egg yolk were from Avanti Polar Lipids, and 3-(*N*-maleimidopropionyl)biotin (MPB) from Molecular Probes. All other materials were reagent grade and obtained from commercial sources.

##### Bacterial Strains and Growth Conditions

*E. coli* strains used were: HB101 (*hsdS20*[r<sup>-</sup><sub>B</sub>m<sup>-</sup><sub>B</sub>], *recA13*, *ara14*, *proA2*, *lacY1*, *galK2*, *rps* [Sm<sup>R</sup>], *xy15*, *mtl1*, *supE44*,  $\lambda^-$ , F<sup>-</sup>), MC1061

( $\Delta$ *lac*[IPOZYA], *araD139*,  $\Delta$ [*ara-leu*]7697, *galU*, *galK*, *rpsL*, *r<sub>K</sub>m<sub>K</sub>*), NO2947 (MC1061, *recA56 srl::Tn10*), and CJ236 (*dut*, *ung*, *thi*, *relA*, *pCJ105* [Cm<sup>R</sup>]). Cells were grown aerobically at 30 or 37 °C in Luria broth or M9 minimal medium supplemented with 0.2% (w/v) casamino acids plus 20 mM glycerol or 30 mM succinate, and the essential nutrients as indicated by the auxotrophic markers. When necessary, the medium was supplemented with carbenicillin (50  $\mu$ g/ml), chloramphenicol (10  $\mu$ g/ml), or erythromycin (300  $\mu$ g/ml).

*S. thermophilus* ST11 and ST11( $\Delta$ *lacS*) were grown semi-anaerobically at 42 °C in (B)elliker broth (Elliker *et al.*, 1956) supplemented with 0.5% beef extract, 20 mM lactose, and with 5  $\mu$ g/ml erythromycin when carrying plasmid pGK13 or derivatives (Poolman *et al.*, 1995b).

##### Plasmid Constructions

The plasmids used for the expression in *E. coli* and *S. thermophilus* and their relevant properties are listed in Table I and oligonucleotides are listed in Table II. For the subcloning of the *lacS* gene with its own ribosome binding site, a new *EcoRI* restriction site was engineered 21-bp upstream of the initiation codon, using site-directed mutagenesis. Single-stranded uracil-containing DNA of plasmid pSKE8 was isolated from *E. coli* CJ236 and served as a template for the annealing of the oligonucleotide primer LacSdp2. Closed-circular DNA was synthesized *in vitro* as described (Kunkel *et al.*, 1987) and the resulting plasmid (pSKE8E) was transformed into *E. coli* MC1061. The new *EcoRI* restriction site was checked by restriction analysis and by nucleotide sequencing of double stranded DNA using the dideoxy chain termination method (Sanger *et al.*, 1977). The 1965-bp *EcoRI-DraI* fragment of plasmid pSKE8E was isolated and ligated into the *EcoRI* and *SmaI* sites of the medium copy number vector pTAQI, yielding plasmid pLS1; the same fragment in the high copy vector pTTQ18, yielded plasmid pLL1. In both plasmids the gene is under control of the isopropyl- $\beta$ -D-thiogalactopyranoside inducible *tac* promoter. For the expression of LacS from the *galP* promoter in plasmid pMTC15,<sup>2</sup> a *NcoI* site was engineered into plasmid pSKE8 that overlaps with the initiation codon of *lacS* (primer *NcoA*) and a second *NcoI* site present in the coding region was removed without changing the amino acid sequence (primer *NcoB*). The resulting plasmid pSKE8N was checked by nucleotide sequencing as described. After digesting plasmid pSKE8N with *EcoRI* and treatment with Klenow enzyme the DNA was digested with *NcoI* and the 2225-bp *NcoI*-blunt end fragment was isolated. Plasmid pMTC15 was partially digested with *HindIII* and the linearized plasmid was treated with Klenow enzyme. Subsequently, the linearized plasmid was isolated and digested with *NcoI* and the 3315-bp *NcoI*-

<sup>2</sup> J. E. Marshall and P. J. F. Henderson, manuscript in preparation.

TABLE II  
Oligonucleotides used for mutagenesis of the lactose transport gene of *S. thermophilus*

Primer	Sequence of (mutagenic) primer (5'-3')	Restriction site
MluI	CAAAAAAGGAG <u>ACGCGT</u> TAATCTATGAAC	New MluI
His-linkS	CGCGATGAGGGTCGTCATCATCACACCACCTCACTGA	
His-linkA	CGCGTCAGTGATGGTGGTGATGATGACGACCCTCAAT	
C320A	TTCTACGGAG <u>CT</u> TATTGCAGTA	Loss of FokI
S384C	CTTTGT <u>G</u> TGTT <u>AGG</u> CCTCTTATT	New StuI
BP27	CACGTGAAGTTGAGTCA	
pEXG	AACAATCTTTGGATCGTTTAAATAAGTTTG	
LacSdp2	AATGTAAC <u>GAATTC</u> AAATAATAGC	New EcoRI
NcoA	GGATGTTCCG <u>CCAT</u> GGAAAAATC	New NcoI
NcoB	AAATTCAAAC <u>CT</u> TGGGTAGTTGG	Loss of NcoI

blunt end fragment was ligated with the 2225-bp *NcoI*-blunt end fragment of pSKE8N. The resulting plasmid was termed pLL11.

#### Engineering of His-6-tag to Wild Type LacS

Site-directed mutagenesis was used to generate a unique *MluI* restriction site (ACGCGT) that overlaps with the stop codon of the *lacS* gene. Single-stranded uracil-containing DNA of plasmid pSKE8 was isolated from *E. coli* CJ236 and served as a template for the annealing of the oligonucleotide primer MluI. The resulting plasmid (pSKE8Mlu) was transformed to *E. coli* MC1061 and the presence of the new *MluI* site was checked by restriction analysis. Subsequently, a linker, consisting of the two annealed oligonucleotides His-link S and His-link A, that codes for a factor Xa cleavage site and a poly(6)histidine tag, was ligated in the *MluI* site of pSKE8Mlu. The insert and flanking regions were checked by nucleotide sequencing as described. The unique *BstEII* and *DraI* sites in the flanking regions were used to isolate a 224-bp fragment, coding for the carboxyl terminus with the His-6-tag, and this fragment was exchanged with the wild type fragment from pSKE8, yielding pSKE8His.

For expression of the His-tagged protein in *S. thermophilus*, the 3824-bp *EcoRI*-*DraI* fragment of pSKE8His was isolated and ligated in the unique *EcoRI* and *EcoRV* sites of shuttle vector pGK13 (Kok *et al.*, 1984), yielding plasmid pGKHHis. *S. thermophilus* strains were transformed as described (Mollet *et al.* 1993).

#### Construction of Cysteine Mutants of LacS-H6

A gene coding for a LacS protein devoid of cysteine residues (wild type contains one cysteine) was made by site-directed mutagenesis using single stranded DNA of plasmid pSKE8 as a template and oligonucleotide C320A as primer. The resulting plasmid was termed pSKE8(C320A). For expression of the mutant protein in *S. thermophilus*, the 2874-bp *EcoRI*-*PstI* fragment of plasmid pSKE8(C320A) was isolated and ligated in the *EcoRI* and *PstI* sites of pGKHHis, thereby replacing the wild type fragment and yielding plasmid pGKH(C320A). Using a two-step polymerase chain reaction approach the serine at position 384 was replaced by a cysteine in the Cys-less LacS-H6. Oligonucleotides S384C and pEXG (Table II) were used to amplify a 810-bp fragment of pSKE8, which, subsequently, was used as a primer in the second polymerase chain reaction with oligonucleotide BP27 and plasmid pGKH(C320A) as template. The expected 1295-bp fragment was digested with *PstI* and *KpnI* and ligated into the *PstI* and *KpnI* sites of pGKH(C320A), thereby replacing the wild type fragment and yielding pGKH(C320A/S384C). The 551-bp *PstI*-*KpnI* fragment of the mutant was checked by nucleotide sequencing.

#### Isolation of Membranes

For the isolation of right-side out membrane vesicles of *E. coli*, cells were disrupted by water lysis as described (Witholt and Boekhout, 1978). For the isolation of inside-out membrane vesicles, cells were lysed by passage through a French pressure cell (20,000 p.s.i.) (Poolman *et al.*, 1983).

Right-side out membrane vesicles of *S. thermophilus* were isolated as described (Otto *et al.*, 1982) with the following modifications: the cell wall was digested with 10 mg/ml lysozyme plus 10 units/ml mutanolysin; DNase and RNase were added to final concentrations of 100 µg/ml each. To remove peripheral membrane proteins as well as cytosolic contaminants, membrane vesicles were extracted with 5 M urea and 6% (w/v) sodium cholate as described (Newman *et al.*, 1981). The membrane preparations were stored in liquid nitrogen.

#### Solubilization

Membranes (3–4 mg of protein/ml) were solubilized in 50 mM potassium phosphate, containing 20% (v/v) glycerol. Optimal solubilization conditions were determined for the different detergents with respect to pH, NaCl and detergent concentration (see "Results"). The suspensions were mixed and after 10–20 min of incubation at 4 °C, the insoluble material was removed by centrifugation (280,000 × *g*, 15 min). The amounts of LacS in the soluble and insoluble fractions were quantitated by Coomassie staining of SDS-PAGE gels and immunoblotting. Conditions routinely used to compare the solubilization efficiency of membranes from *E. coli* and *S. thermophilus* involved the use of the following medium: 50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 20% (v/v) glycerol plus detergent.

#### Immunoblotting

The amount of wild type and LacS-H6 in the different samples was estimated by immunodetection with antibodies raised against synthetic peptides and directed against the NH<sub>2</sub> or COOH terminus of the wild type protein (Poolman *et al.*, 1995b). The membrane proteins were separated by SDS-PAGE (12.5% polyacrylamide) and transferred to polyvinylidene difluoride membranes by semi-dry electrophoretic blotting. Detection, using the Western-Light™ chemiluminescence detection kit with CSPD™ as a substrate, was performed as recommended by the manufacturer (Tropix Inc.).

#### Purification of LacS-H6

The solubilized membrane proteins were mixed and incubated for 30 min with Ni-NTA resin (~4 mg of LacS/ml of resin) that was equilibrated with buffer A (50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, 0.2 mg/ml *E. coli* lipids plus detergent at a concentration of 5 × CMC) plus 10 mM imidazole. The column material was poured into a Bio-Spin column (Bio-Rad) and washed with 10 column volumes of buffer A containing 25 mM imidazole. The protein was eluted with buffer A, pH 7, containing 200 mM imidazole. The purification was carried out at 4 °C with sterile solutions. Fractions from the Ni column were diluted 10 times with buffer B (50 mM potassium phosphate, pH 7, 10% (v/v) glycerol, 0.2 mg/ml *E. coli* lipids plus detergent at a concentration of 5 × CMC) and loaded onto a Mono Q column (HR 5/5, Pharmacia Biotech). The proteins were eluted with a NaCl gradient running from 0 to 750 mM in buffer B.

#### Reconstitution of LacS-H6

For the reconstitution of purified protein, liposomes were prepared from acetone/ether washed *E. coli* lipids and L-α-phosphatidylcholine from egg yolk in a ratio of 3:1 (w/w). Unilamellar vesicles with relatively homogenous size were made by dialysis of octyl glucoside dissolved lipids (Driessen and Konings, 1993), followed by freezing in liquid nitrogen, slow thawing at room temperature, and extrusion through a 400-nm polycarbonate filter (Mayer *et al.*, 1986). Subsequently, the liposomes were diluted to 4 mg of PL/ml and titrated with detergent, which was followed by measuring the absorbance at 540 nm using a SLM-Aminco spectrophotometer (Paternostre *et al.*, 1988). The liposomes titrated with detergent were mixed with the purified protein in a ~100:1 ratio (w/w), unless specified otherwise, and incubated for 30 min at 20 °C under gentle agitation. For the removal of detergent, polystyrene beads (Bio-Beads SM-2; extensively washed with methanol and H<sub>2</sub>O) were added at a wet weight of 80 mg/ml (liposomes suspension of 4 mg of PL/ml) and the sample was incubated for another 2 h at room temperature. Fresh polystyrene beads were added twice, after removal of Bio-Beads by filtration on glass silk, and the samples were incubated

at 4 °C for 3 h and overnight, respectively. The proteoliposomes were washed with 50 mM potassium phosphate, pH 7.0, harvested by centrifugation, and stored in liquid nitrogen.

#### Orientation of the Reconstituted LacS Protein

The mutant proteins LacS(C320A) and LacS(C320A/S384C) were expressed in *S. thermophilus* and purified using Triton X-100, and reconstituted into liposomes titrated with a low concentration (1.8 mM) of the detergent. Proteoliposomes were washed twice and resuspended to a final protein concentration of ~0.5 mg/ml in potassium phosphate, pH 7.5, 100 mM NaCl, and labeled from the outside with 300  $\mu$ M MPB at 30 °C. For the outside and inside labeling, the proteoliposome samples were labeled following sonication with a microtip at an output of 4  $\mu$ m (peak to peak) for 3 s in the presence of MPB (Bayer *et al.*, 1985). The labeling reactions were stopped by the addition of 10 mM dithiothreitol, and analyzed by Western blotting and detection with streptavidin/alkaline phosphatase and CSPD<sup>TM</sup> as a substrate.

#### Transport Assays

(i) *Lactose Uptake in E. coli*—Cells were grown overnight in Luria broth, washed, and resuspended in KPM (50 mM potassium phosphate, pH 7.0, plus 2 mM MgSO<sub>4</sub>) supplemented with 10 mM D-Li-lactate. After 2 min of energization in the presence of oxygen, [<sup>14</sup>C]lactose was added to a final concentration of 50  $\mu$ M and uptake was assayed for different time intervals. The reaction was stopped by diluting the mixture with 2 ml of ice-cold 0.1 M LiCl and the cells were collected on 0.45- $\mu$ m cellulose nitrate filters. The filters were subsequently washed with another 2 ml of the LiCl solution.

(ii) *TMG Counterflow in S. thermophilus*—Cells grown in Belliker broth with 0.5% lactose or 0.5% sucrose were washed and resuspended in KPM. The cells were equilibrated with 2 mM TMG for 1 h at 42 °C and concentrated by centrifugation. The concentrated cell suspensions were diluted 40-fold into 100 mM potassium phosphate, pH 6.6, 2 mM MgSO<sub>4</sub>, containing [<sup>14</sup>C]TMG at a final concentration of 57  $\mu$ M. The reaction was stopped as described above (i).

(iii) *Lactose Counterflow in Proteoliposomes*—Proteoliposomes were resuspended in KPM plus 10 mM lactose and frozen in 1-ml aliquots in liquid nitrogen. After thawing the samples at room temperature, the liposomes were extruded through a 400-nm filter to obtain unilamellar vesicles of relatively homogenous size (Mayer *et al.*, 1986). After centrifugation, aliquots (1–2  $\mu$ l) of concentrated membrane suspensions were diluted into 200  $\mu$ l of KPM containing 10  $\mu$ M [<sup>14</sup>C]lactose; this yields a final lactose concentration of 60–110  $\mu$ M, unless indicated otherwise. The reaction was stopped as described above (i).

(iv)  *$\Delta p$ -driven Lactose Uptake in Proteoliposomes*—The uptakes driven by artificially imposed diffusion potentials were performed as described (Foucaud and Poolman, 1992). Proteoliposomes were loaded with 20 mM potassium phosphate, pH 7.0, 100 mM KAc plus 2 mM MgSO<sub>4</sub> by freeze/thaw/extrusion as described above (iii). Aliquots of concentrated membrane suspensions (~4 mg of protein/ml) were diluted 100-fold into 120 mM NaPipes, pH 7.0, 2 mM MgSO<sub>4</sub>, containing 0.5  $\mu$ M valinomycin and [<sup>14</sup>C]lactose at varying concentrations (see figure legends). The reaction was stopped as described above (i).

#### Miscellaneous

Protein was assayed according to Lowry *et al.* (1951) in the presence of 0.5% SDS using bovine serum albumin as a standard. DNA manipulations were carried out according to Sambrook *et al.* (1989). The NH<sub>2</sub>-terminal sequence was determined with an automated sequence apparatus (Model 477A, Applied Biosystems) and liberated amino acids were analyzed by high performance liquid chromatography (Eurosequence B.V., Groningen, the Netherlands).

#### RESULTS

*Overexpression of LacS in E. coli*—Several plasmid constructs were made to amplify LacS in *E. coli* (Table I). Expression systems using high and low copy number plasmids with inducible (*tac*) and constitutive (*galP* and *lacS*) promoters were tested under varying growth conditions, and in various hosts (*e.g.* NO2947, HB101, and MC1061) and the expression levels were evaluated by transport assays and SDS-PAGE. The highest levels of LacS protein in the membrane were obtained with its own promoter using plasmid pSKE8 in strain NO2947 grown at 37 °C in minimal medium with glycerol as a carbon and energy source (Fig. 1, lanes 3 and 7). Like most integral

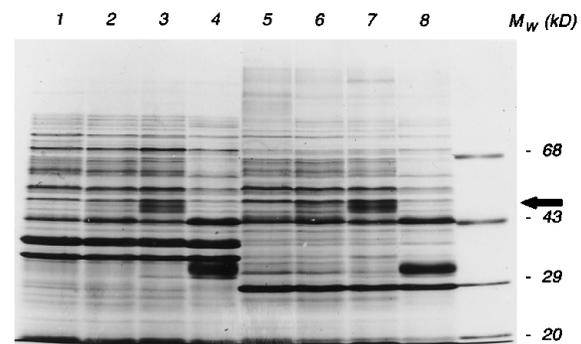


FIG. 1. Amplification of the lactose transport protein of *S. thermophilus* in *E. coli*. Coomassie Brilliant Blue-stained SDS-PAGE gel (10%) with samples of right-side out membrane vesicles (50  $\mu$ g of protein/lane); lanes 1 and 5, *E. coli* NO2947/pTTQ18; lanes 2 and 6, *E. coli* NO2947/pLL01; lanes 3 and 7, *E. coli* NO2947/pSKE8; lanes 4 and 8, *E. coli* NO2947/pWJL24.<sup>3</sup> Cells were grown in minimal medium with 20 mM glycerol at 37 °C; for the pTTQ derivatives, membranes were isolated after overnight induction with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside. Protein samples of lanes 1–4 were solubilized for 20 min at room temperature; the samples of lanes 5–8 were boiled for 10 min. The arrow indicates the LacS protein.

membrane proteins, LacS is migrating at a lower apparent molecular mass than predicted from the deduced amino acid sequence, *i.e.* ~51 instead of 69 kDa. The highest expression with an inducible promoter was obtained with the *tac* promoter in plasmid pLL01 in strain NO2947, also grown in minimal medium with glycerol and after overnight induction with 2 mM isopropyl- $\beta$ -D-thiogalactopyranoside (Fig. 1, lanes 2 and 6); the plasmid control pTTQ18 is shown in lanes 1 and 5. With *lacS* under control of its own promoter, the level of LacS expression in NO2947/pSKE8 was approximately 8% of total membrane protein as determined by densitometry. On the basis of immunoblots using an antibody directed against the COOH terminus of LacS, the expression in NO2947/pLL01 was about 2-fold lower than in NO2947/pSKE8 (data not shown). The overexpression of the *E. coli* glucuronide transport protein (GusB) from the *tac* promoter in pTTQ18 is shown for comparison (Fig. 1, lanes 4 and 8) (~25% of total membrane protein).<sup>3</sup> The initial rate of lactose uptake in whole cells (at 50  $\mu$ M, final concentration) was 6 nmol/min/mg of protein for NO2947/pSKE8 as compared to 2.6 nmol/min/mg of protein for NO2947/pLL01, which correlate with the expression levels of LacS in the membranes. Accumulation of lactose is not detectable in *E. coli* NO2947/pTTQ18 (control).

*Overexpression of LacS in S. thermophilus*—For the amplification of LacS in a homologous expression system, the *lacS* gene with about 2 kilobases of 5' DNA from the chromosome of *S. thermophilus* was ligated into plasmid pGK13, a shuttle vector that replicates both in *E. coli* and *S. thermophilus*. The plasmid, pGKGS8, was transformed into *S. thermophilus* ST11 and ST11( $\Delta$ lacS) and the cells were grown in Belliker plus lactose as carbon and energy source and to induce the *lacS* promoter. The transport activity of both strains was compared to strains carrying the plasmid control pGK13 (Fig. 2). TMG counterflow rates were highest in strains containing pGKGS8; the additional copy of the *lacS* gene present on the chromosome of the wild type ST11 did not lead to a higher transport activity, indicating that the maximum attainable level of expression was reached. It was difficult to determine the initial rates of TMG uptake accurately, but, in various trials, the transport activity of ST11( $\Delta$ lacS)/pGKGS8 (or ST11/pGKGS8) was at least 2–3-fold higher than ST11/pGK13. Similar results were

<sup>3</sup> W.-J. Liang, J. Knol, B. Poolman, and P. J. F. Henderson, manuscript in preparation.

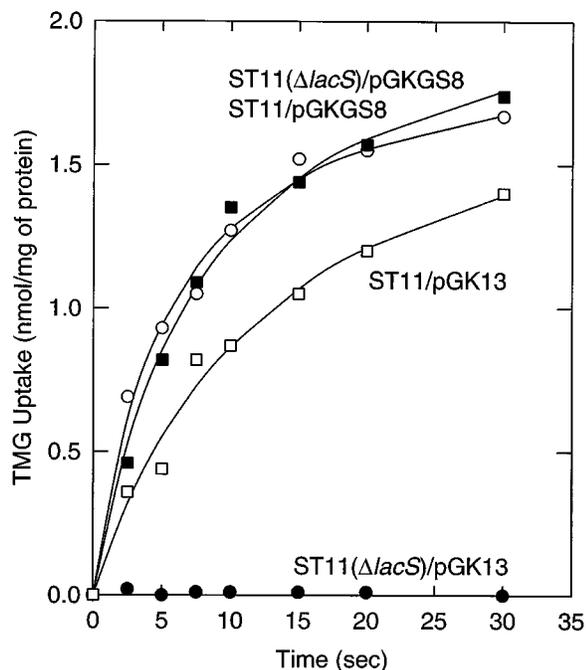


FIG. 2. TMG transport in *S. thermophilus*: effect of gene dosage. *S. thermophilus* ST11/pGK13, ST11/pGKGS8, ST11( $\Delta$ lacS)/pGKGS8, and ST11( $\Delta$ lacS)/pGK13 cells grown in Belliker broth with 0.5% (w/v) lactose (or sucrose in the case of ST11( $\Delta$ lacS)/pGK13) were harvested and washed with KPM buffer. The counterflow assay is described under "Experimental Procedures," transport assays (ii). The assay temperature was 23 °C; the final protein concentrations were 0.8–1.0 mg/ml.

obtained with LacS-H6 (histidine tag engineered to the COOH terminus) instead of wild type LacS. To quantitate the levels of expression of LacS, right-side out membrane vesicles of *S. thermophilus* were analyzed by SDS-PAGE before and after extraction with 5 M urea and 6% (w/v) sodium cholate. Fig. 3 shows the expression levels in *S. thermophilus* of LacS-H6 using pGKGS8 as expression vector. These levels varied between 25 and 30% of total membrane protein in urea/cholate extracted membranes, and were independent of whether the His<sup>6</sup>-tag was engineered to the COOH terminus of the protein (data not shown). Lanes 1 and 4 show a strain in which the *lacS* gene was present on the chromosome rather than on the plasmid (the *lacS* genes of strain A147 and ST11 are identical). The reduced expression level corresponds with the lower activity of TMG counterflow in ST11/pGK13 as compared to ST11( $\Delta$ lacS)/pGKGS8 (Fig. 2). Also, the LacS protein without the IIA-domain (LacS $\Delta$ IIA) could be overexpressed to high levels (lanes 3 and 6). LacS-H6 is migrating at a slightly higher molecular weight compared to the wild type as a result of 10 additional amino acids, *i.e.* IEGRHHHHHH. Electron microscopy of negatively-stained cells showed that overexpression did not lead to the formation of inclusion bodies either in *E. coli* or *S. thermophilus* (data not shown). Moreover, subcellular structures such as stacked membranes or invaginations were not seen in electron micrographs of cells overexpressing LacS.

**Solubilization**—The detergents octyl glucoside (0.8–2.0%), *n*-dodecyl- $\beta$ -D-maltoside (0.1–1.0%), C<sub>12</sub>E<sub>8</sub> (0.5 to 8%), and Triton X-100 (0.1 to 2%) were tested for their ability to solubilize LacS from *S. thermophilus* and *E. coli* membranes. Solubilizations were carried out at pH 6.0–8.0 with NaCl concentrations varying from 0 to 600 mM, and protein concentrations ranging from 1 to 5 mg/ml. The degree of solubilization was similar at every pH value tested (data not shown). Since the protein is subsequently used for purification on a Nickel column, a pH of 8.0 was used in most experiments and the concentration of the

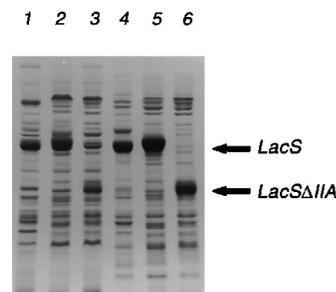


FIG. 3. Amplification of LacS in *S. thermophilus*. Coomassie Brilliant Blue-stained SDS-PAGE gel (12.5%) with samples of right-side out membrane vesicles (80  $\mu$ g of protein/lane); lanes 1 and 4, *S. thermophilus* ST11/pGK13; lanes 2 and 5, *S. thermophilus* ST11( $\Delta$ lacS)/pGKHis; lanes 3 and 6, *S. thermophilus* ST11( $\Delta$ lacS)/pGKGS8 (*lacS* $\Delta$ 160). Cells were grown in Belliker with 0.5% lactose at 42 °C. Samples 1–3, right-side out membrane vesicles; samples 4–6, right-side out membrane vesicles that were extracted with urea/cholate. The arrows indicate the LacS proteins.

protein was kept relatively high at 4 mg/ml (Pourcher *et al.*, 1995). For the detergents used the effect of NaCl was significant; increasing concentrations of NaCl decreased the efficiency of solubilization and the protein seemed to aggregate in the presence of high concentrations of salt (600 mM). This was also observed by SDS-PAGE where the protein runs as higher order aggregates, especially when C<sub>12</sub>E<sub>8</sub> was used as detergent (data not shown). Irrespective of whether octyl glucoside, *n*-dodecyl- $\beta$ -D-maltoside, C<sub>12</sub>E<sub>8</sub>, or Triton X-100 was used, more than 95% of LacS and ~60% of total protein were solubilized from membranes of *S. thermophilus* (Table III). For the comparison of the solubilization of LacS from membranes of *E. coli* and *S. thermophilus*, buffer C (50 mM potassium phosphate, pH 8.0, 100 mM NaCl, 20% of glycerol) and total membrane protein concentrations of 4 mg/ml were used. Under these conditions the efficiency of solubilization of both types of membranes was significantly different (Table III). Further testing of solubilization conditions with the membranes from *E. coli* did not lead to an improvement.

**Purification**—Since the expression levels of LacS in *S. thermophilus* were highest and the solubilization was far more efficient than in *E. coli*, right-side out membranes from *S. thermophilus* were used as starting material for the purification of the LacS-H6 protein (Fig. 4, lane 1). The His-tagged protein could almost be purified to homogeneity in a single step using nickel chelate affinity chromatography (Fig. 4, lane 3), even when the urea-cholate extraction step was omitted (Fig. 4, lane 2). Most contaminants were removed by washing the column with 25 mM imidazole, at medium ion strength (100 mM NaCl, 50 mM potassium phosphate, pH 8.0, detergent (at a concentration of ~5  $\times$  CMC) and 10% glycerol). The protein was eluted with 50 mM potassium phosphate, pH 7.0, 100 mM NaCl, 10% glycerol, detergent as above plus 200 mM imidazole, and peak fractions contained LacS at a concentration of 1.0–1.5 mg/ml and with a purity of >95%. The minor contaminants were also binding to the Ni-affinity resin when a total membrane extract, containing wild type LacS rather than LacS-H6, was applied to the column (data not shown). This indicates that these contaminants are not associated with LacS *per se*. Further purification was achieved by anion exchange chromatography on a Mono Q column (Fig. 4, lane 4). The protein was eluted at a NaCl concentration of ~500 mM. From 1 liter of cells, grown in Belliker medium plus lactose to an OD<sub>660</sub> = 0.8–1.0, 40–50 mg of crude membrane protein was obtained, from which 4–5 mg of LacS was purified. The NH<sub>2</sub>-terminal sequence of the purified protein was Met-Glu-X-Ser-Lys-Gly-Gln-Met-Lys-Ser-Arg-, which is identical to the deduced amino

TABLE III

Percentages of LacS protein solubilized from membranes of *E. coli* and *S. thermophilus* using different detergents

Inside out membrane vesicles of *E. coli* and right-side out membrane vesicles of *S. thermophilus* were resuspended in buffer C at 4 mg of protein/ml, and the different detergents were added at the concentration indicated. After mixing, the proteins were solubilized for 20 min on ice. The insoluble material was pelleted at  $500,000 \times g$  for 15 min, and the soluble and insoluble fractions were analysed by Coomassie-stained SDS-PAGE and immunoblotting.

	<i>E. coli</i>	<i>S. thermophilus</i>
Sodium cholate (0–6% w/v)	< 5%	< 5%
Octyl- $\beta$ -D-glucoside (1% w/v)	<70%	>95%
Dodecyl- $\beta$ -D-maltoside (0.5% w/v)	<70%	>95%
C <sub>12</sub> E <sub>8</sub> (2% w/v)	<50%	>95%
Triton X-100 (0.5% w/v)	<40%	>95%

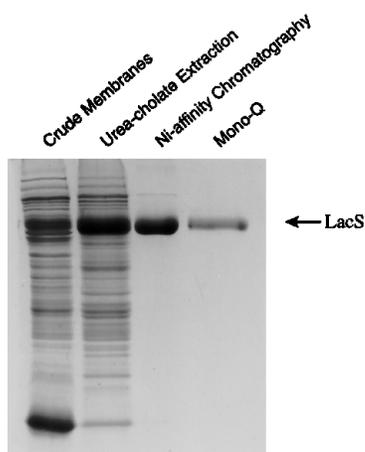


FIG. 4. Purification of the LacS protein of *S. thermophilus*. Coomassie Brilliant Blue-stained SDS-PAGE gel (12.5%). Lane 1, right-side out membrane vesicles of *S. thermophilus* ST11( $\Delta$ lacS)/pGKHis (20  $\mu$ g of protein); lane 2, urea/cholate extracted membranes of *S. thermophilus* (20  $\mu$ g of protein); lane 3, LacS-H6 after nickel affinity chromatography (~10  $\mu$ g of protein); lane 4, LacS-H6 after anion exchange chromatography (~2  $\mu$ g of protein).

acid sequence (*X* being a Lys; Poolman *et al.* (1989)). Apparently, the amino terminus still possesses the methionine encoded by the initiation codon.

**Reconstitution**—Detergent-mediated reconstitution of LacS from solubilized membranes of *S. thermophilus* has been successful using octyl glucoside and detergent removal by dilution or dialysis (Foucaud and Poolman, 1992). However, when LacS protein was purified in the presence of octyl glucoside, no transport activity was observed upon reconstitution into proteoliposomes, using detergent dilution, dialysis, or adsorption to polystyrene beads to remove the octyl glucoside. Also, the first attempts using C<sub>12</sub>E<sub>8</sub> or *n*-dodecyl- $\beta$ -D-maltoside gave low transport activities and a low reproducibility. Therefore, the reconstitution process had to be examined more precisely, which was done using strategies described by Rigaud *et al.* (1988). Since membrane reconstitution is dependent on the type of detergent used, lipid to protein ratios, and the physical state of the lipid-detergent mixture, these parameters were studied in detail. Rather than using dispersed lipids, preformed liposomes formed the starting material for our reconstitutions, unless indicated otherwise. The liposomes are composed of *E. coli* PL and egg PC in a ratio of 3:1 and were formed by detergent dilution, followed by freeze/thaw/extrusion as described under "Experimental Procedures." Although the lipid requirement of LacS has not been studied in detail, liposomes prepared from mixtures of *E. coli* PL and egg PC yielded the highest activities as was observed for other transport proteins of (lactic acid) bacteria (Driessen *et al.*, 1988). To follow the

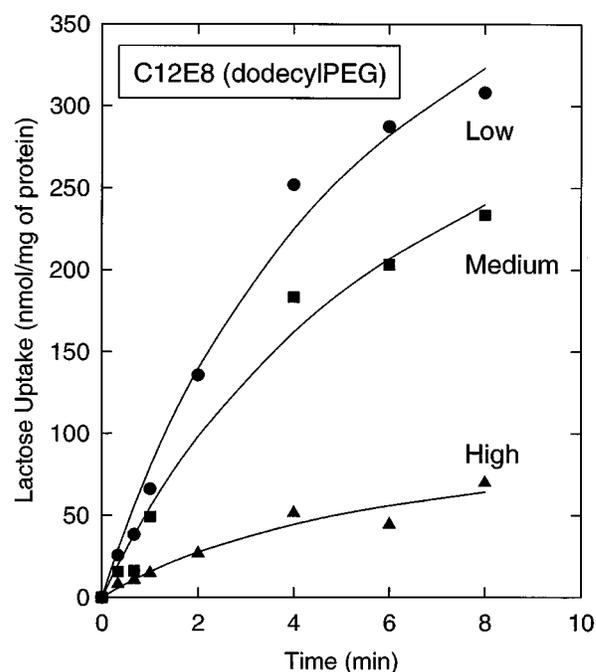
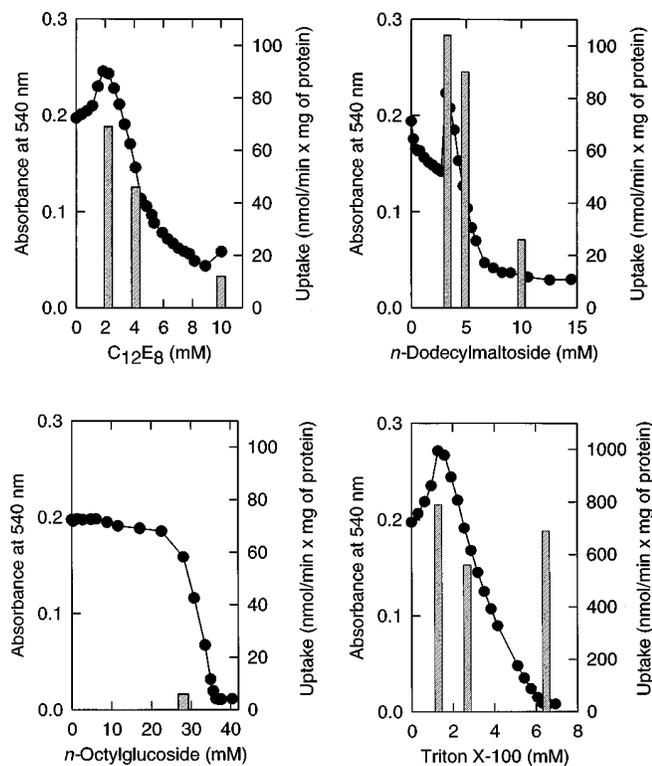


FIG. 5. Counterflow activity in proteoliposomes of LacS-H6 purified and reconstituted with C<sub>12</sub>E<sub>8</sub>. Purified LacS-H6 was added to preformed liposomes titrated with low (2.2 mM), medium (4.1 mM), and high (10.1 mM) concentrations of C<sub>12</sub>E<sub>8</sub>. After removal of detergent, the proteoliposomes were washed with potassium phosphate, pH 7.0, plus 2 mM MgSO<sub>4</sub> (KPM), and loaded with 10 mM lactose by freeze/thaw/extrusion. The counterflow reaction was started by diluting concentrated proteoliposome suspensions (~1–5 mg of protein/ml) 200-fold into KPM containing [<sup>14</sup>C]lactose; the final lactose concentration was 60  $\mu$ M. The assay temperature was 30 °C.

physical state of the liposomes the absorbance at 540 nm was measured upon stepwise addition of detergent. At different stages of the titration curve, purified LacS protein was added to the lipid/detergent mixture and then the detergent was removed at a controlled rate with the use of polystyrene beads (Holloway, 1973).

An example of reconstitution of LacS into preformed liposomes at different detergent concentrations is shown for C<sub>12</sub>E<sub>8</sub> (Fig. 5); low, medium, and high correspond to 2.2, 4.1, and 10.1 mM C<sub>12</sub>E<sub>8</sub>, respectively. The uptake of lactose was measured in a counterflow assay upon equilibration of the proteoliposomes with lactose and 100-fold dilution into buffer containing [<sup>14</sup>C]lactose. The highest activity was obtained when liposomes were titrated with a low concentration (2.2 mM) of the C<sub>12</sub>E<sub>8</sub> (Figs. 5 and 6A). The absorbance at 540 nm is maximal at this concentration, suggesting that the liposomes are saturated with the detergent but are still in the lamellar state. Using liposomes that were partially disintegrated, the activity was reduced by about 30% and using mixed micelles of lipid and detergent the activity was even reduced by 80%. This suggests that the LacS protein is most efficiently inserted by integration or fusion of the detergent/protein micelle with the detergent saturated liposomes. The tightness of the proteoliposomes is shown by the increase of lactose uptake up to at least 8 min.

Using the same approach, the optimal conditions for reconstitution were also determined for other detergents. *n*-Dodecyl- $\beta$ -D-maltoside shows a titration curve in which the absorbance of the liposome suspension is decreasing initially, after which a steep increase in absorbance is observed. Since equilibration of *n*-dodecyl- $\beta$ -D-maltoside and liposomes is slow, the solubilization process cannot be analyzed accurately (see also Rigaud and Pitard (1995)). The counterflow activity was again measured at detergent concentrations at which, on the basis of the

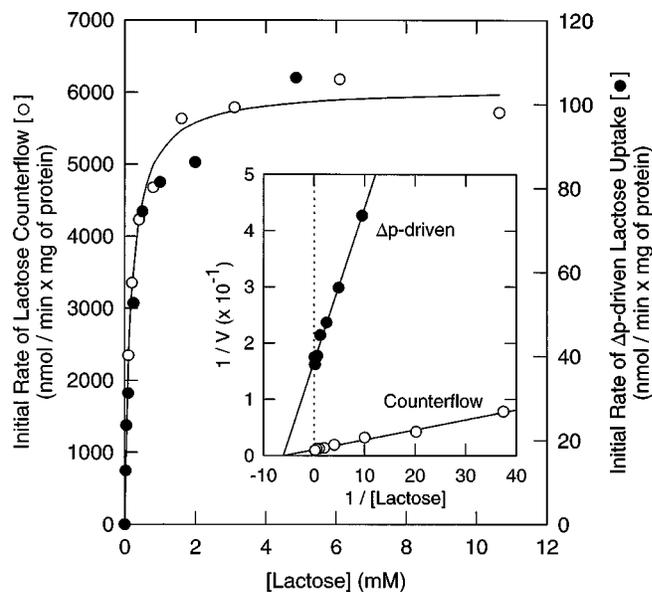


**FIG. 6. Lactose counterflow activities in proteoliposomes with LacS-H6 protein purified and reconstituted using different detergents.** For the reconstitution, purified LacS protein was added to preformed liposomes titrated with the different detergents at the concentrations indicated. After removal of detergent and washing of the proteoliposomes with KPM, the initial rates of counterflow (shaded bars) were estimated from transport curves as shown in Fig. 5. The state of the liposomes was followed by measuring the absorbance at 540 nm upon stepwise addition of detergent (●).

absorbance changes, the liposomes are predicted to be in the lamellar, the lamellar-micellar state (~50% decrease  $A_{540}$ ), and the micellar state. Fig. 6 shows a similar *n*-dodecyl- $\beta$ -D-maltoside concentration dependence of the lactose uptake rate as was observed for  $C_{12}E_8$  (Figs. 5 and 6). The transport activity was highest at the *n*-dodecyl- $\beta$ -D-maltoside concentration at which the liposomes were destabilized but most likely still in the lamellar state. These observations explain the low activities in the first reconstitution experiments with *n*-dodecyl- $\beta$ -D-maltoside and  $C_{12}E_8$ , because these were always performed with mixed micelles of lipid and detergent.

Using octyl glucoside the purified LacS protein could not be functionally reconstituted (Fig. 6). Since LacS can be reconstituted functionally from total membrane protein extracts obtained with octyl glucoside, this detergent seems to inactivate the protein during the purification. The presence of carrier substrates (galactosides) throughout the solubilization/purification/reconstitution did not improve the LacS activity in the proteoliposomes, *i.e.* when octyl glucoside was used. The highest transport activities were obtained using Triton X-100 as a detergent and initial uptake activities of up to 800 nmol/min/mg of protein were found that depended relatively little on the physical state of the liposomes, *i.e.* lamellar or micellar (Fig. 6).

The counterflow activities in the proteoliposomes were not only dependent on the physical state of the liposomes at the beginning of reconstitution but also on the glycerol concentration, the NaCl concentration, lipid to protein ratio, temperature, and rate of detergent removal. Glycerol concentrations above 1% (v/v) at the beginning of the reconstitution reduced



**FIG. 7. Kinetics of lactose uptake.** Counterflow and  $\Delta p$ -driven lactose transport in proteoliposomes reconstituted with purified LacS-H6, using Triton X-100 as detergent for the solubilization/purification and reconstitution. The counterflow assay was performed as described in the legend to Fig. 6. For the  $\Delta p$ -driven lactose uptake reaction, proteoliposomes were loaded with 20 mM potassium phosphate, pH 7.0, 100 mM KAc plus 2 mM  $MgSO_4$  by freeze/thaw/extrusion. Aliquots of concentrated proteoliposomes were diluted 100-fold into 120 mM NaPipes, pH 7.0, plus 2 mM  $MgSO_4$ , containing 0.5  $\mu M$  valinomycin plus [ $^{14}C$ ]lactose at the concentration indicated. The data were fitted to Michaelis-Menten and replotted as Lineweaver-Burk (*inset*).

the counterflow activities of the proteoliposomes; 600 mM NaCl also reduced the activity probably due to aggregation of the protein; lipid to protein ratios (weight/weight) of 100–200 were optimal (data not shown); and higher transport activities were obtained when LacS protein was allowed to insert into the detergent-destabilized liposomes at 20 rather than at 4 °C and when the detergent was removed at a controlled rate by adding small amounts of polystyrene beads (<100 mg/ml liposome suspension). Less important seemed to be the pH (between 6 and 8) and the presence of lipids during the solubilization. Reconstitution conditions that gave the highest activity for Triton X-100 solubilized and purified LacS include: elution of the LacS protein from the nickel column in 50 mM potassium phosphate, pH 7.0, 100 mM NaCl, 10% of glycerol, 0.2 mg/ml lipid, 0.1% (w/v) Triton X-100, and 200 mM imidazole. 10-Fold dilution of the protein samples into the liposome suspensions (4 mg PL/ml in potassium phosphate, pH 7.0) that were equilibrated with 1.8 mM Triton X-100. Equilibration of the protein/lipid/detergent suspension was for 30 min at 20 °C. Addition of polystyrene beads (Bio-Beads SM-2) at 80 mg/ml and incubation of the suspension for another 2 h at 20 °C; the polystyrene beads were replaced twice and detergent was removed overnight at 4 °C.

Prolonged incubations with the polystyrene beads did not further increase the activity or the integrity of the proteoliposomes. Although studied in less detail, these conditions also seem optimal when *n*-dodecyl- $\beta$ -D-maltoside or  $C_{12}E_8$  are used as detergent (data not shown).

**Transport Kinetics of Purified and Reconstituted LacS-H6 Protein**—The kinetic parameters for both lactose counterflow and  $\Delta p$ -driven lactose uptake were determined in proteoliposomes, prepared from the purified LacS protein in Triton X-100. For the counterflow assay, the proteoliposomes were loaded with 10 mM lactose by freeze/thaw/extrusion and diluted into buffer with radiolabeled lactose. In Fig. 7, the initial rates

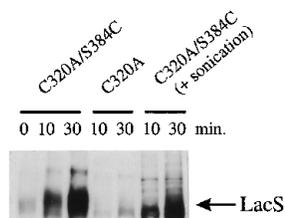


FIG. 8. Labeling of reconstituted LacS(C320A/S384C)-H6 with MPB. Proteoliposomes with LacS(C320A/S384C)-H6 or LacS(C320A)-H6, using 1.5 mM Triton X-100 for the reconstitution, were washed and resuspended in potassium phosphate, pH 7.5. For the outside labeling (shown for LacS(C320A/S384C) and LacS(C320A)), MPB was added to a final concentration of 300  $\mu\text{M}$  and the reaction was performed at 30  $^{\circ}\text{C}$  for 0, 10, and 30 min. For inside and outside labeling (only shown for LacS(C320A/S384C)), the samples were sonicated at zero time. Proteins were separated on SDS-PAGE (12.5%) gels, transferred to polyvinylidene difluoride membranes, and the MPB-labeled protein was detected with streptavidin/alkaline phosphatase using CSPD<sup>TM</sup> as a substrate.

of counterflow are plotted against the external concentration of lactose. The apparent  $K_m$  was determined at  $164 \pm 15 \mu\text{M}$  with a  $V_{\text{max}}$  of  $6.0 \times 10^3 \text{ nmol/min} \times \text{mg}$  of protein. This reflects a turnover number of  $7 \text{ s}^{-1}$ . For the  $\Delta p$ -driven uptake of lactose, the apparent affinity constant for lactose was the same,  $169 \pm 10 \mu\text{M}$ , but the  $V_{\text{max}}$  was 60-fold lower, *i.e.*  $95 \text{ nmol/min} \times \text{mg}$  of protein (turnover number of  $0.11 \text{ s}^{-1}$ ). This indicates that the exchange (counterflow) mode of transport is much more rapid than  $\Delta p$ -driven uptake, which is consistent with the suggestion that *in vivo* LacS mediates lactose/galactose exchange (Poolman, 1990). The  $K_m^{\text{app}}$  for lactose counterflow in proteoliposomes, prepared from LacS protein in *n*-dodecyl- $\beta$ -D-maltoside, was  $187 \mu\text{M}$ ; the  $V_{\text{max}}$  was  $1.0 \times 10^3 \text{ nmol/min} \times \text{mg}$  of protein, which is 6-fold lower than for the Triton X-100 purified enzyme (data not shown).

**Orientation of Reconstituted LacS Protein**—Since the LacS protein contains a large hydrophilic domain (IIA), it is possible that the LacS protein incorporates unidirectionally, *i.e.* with an inside-out orientation, into the detergent-destabilized liposomes. To test this hypothesis a single cysteine mutant was constructed with a cysteine (Cys-384) located in a putative cytoplasmic loop of the protein. This mutant LacS(C320A/S384C), in which the wild type cysteine was replaced by an alanine (C320A), was expressed in *S. thermophilus*. The mutant protein still allows *S. thermophilus* to grow normally on Belliker with lactose as sole source of carbon and energy. The His-tagged protein was purified and reconstituted into proteoliposomes titrated with a low concentration of Triton X-100 (1.8 mM), and the accessibility of the cysteine for the membrane impermeable MPB probe was tested (Fig. 8). The labeling of the LacS(C320A/S384C) protein was complete when MPB was present on the outside. Sonication of the proteoliposomes, which makes both the inside and the outside accessible to MPB, did not enhance the rate and extent of labeling. The negative control, LacS(C320A), showed a minor, aspecific, reaction with MPB. The apparent outside orientation of the cysteine in proteoliposomes containing LacS(S384C) suggests that the protein is indeed inserted unidirectionally. The location of Cys-384 in a putative cytoplasmic loop and the membrane impermeability of MPB was shown by comparing the labeling of LacS(C320A/S384C) in right-side out and inside out membrane vesicles.<sup>4</sup>

#### DISCUSSION

The lactose transport protein of *S. thermophilus* (LacS) has been overexpressed using *E. coli* and *S. thermophilus* as expression hosts. Surprisingly, the highest expression in *E. coli* was obtained when the protein was expressed from the *lacS*

promoter and with the streptococcal Shine-Dalgarno sequences. The levels of LacS in membranes of lactose-grown wild type *S. thermophilus* cells are higher than the highest amplification reached in *E. coli*. These expression levels could even be increased by expressing the LacS protein from a plasmid carrying the *lacS* gene behind the *lacS* promoter (ST11 $\Delta$ lacS/pGKGS8). When the protein was expressed from the plasmid in a wild type background, with an additional copy of the gene on the chromosome (ST11/pGKGS8), the expression was not increased further. The increase in expression level of LacS in ST11 $\Delta$ lacS/pGKGS8 relative to ST11/pGK13 conforms with a 2–3-fold increased rate of TMG counterflow. Although the *S. thermophilus* expression system is preferred for the isolation and purification of wild type and mutant LacS proteins, the levels of expression in *E. coli* are sufficient to perform the initial screening of mutants and to purify mutant proteins that cannot be expressed in *S. thermophilus*. Besides the higher expression in *S. thermophilus*, purification of the LacS protein from this organism has the additional advantage that the membranes of this Gram-positive organism are more efficiently solubilized, with a whole range of detergents, than those of *E. coli*.

Since the LacS protein could be reconstituted from octyl glucoside solubilized membranes of *S. thermophilus* (Foucaud and Poolman, 1992), our first attempts to reconstitute the purified protein were performed with octyl glucoside as detergent. Although several reconstitution parameters were varied, we were unable to obtain active proteoliposomes. On the basis of SDS-PAGE, LacS seems to aggregate when purified in octyl glucoside which may prevent functional insertion into the lipid bilayer. It has been suggested that octyl glucoside is able to penetrate into the hydrophobic parts of a membrane protein, thereby partially unfolding and destabilizing the polypeptide (Lund *et al.*, 1989; Rigaud and Pitard, 1995).

To optimize the reconstitution, the strategy of Rigaud *et al.* (1988) was followed. The liposomes, however, were prepared differently and made from total *E. coli* phospholipids plus egg PC in a 3:1 ratio. The liposomes were made by freeze/thaw/extrusion to obtain relatively large and homogeneous vesicles. The physical state of the liposomes during the titration with the detergent was followed by measuring the absorbance at 540 nm. Titration curves obtained with Triton X-100 and octyl glucoside are comparable with the curves that have been published for liposomes composed of egg PC/phosphatidic acid in a 9:1 ratio, which were made by reverse-phase evaporation (Paternostre *et al.*, 1988). Titrations with *n*-dodecyl- $\beta$ -D-maltoside and  $\text{C}_{12}\text{E}_8$  show a similar behavior as Triton X-100. The increase in  $A_{540}$  is probably reflecting the swelling of the liposomes due to the partitioning of detergent molecules in the membrane, and the subsequent decrease most likely reflects the disintegration of the liposomes. When liposomes are titrated with *n*-dodecyl- $\beta$ -D-maltoside, however, the steady state absorbance values are reached very slowly ( $>30 \text{ min}$ ), whereas the absorbance changes by the other detergents take place rapidly ( $<2 \text{ min}$ ). It is possible that *n*-dodecyl- $\beta$ -D-maltoside first binds to the interface region of the liposomes and then slowly integrates into the hydrocarbon region by rearrangement of detergent and lipid molecules. The concentration of octyl glucoside at which the liposomes are destabilizing (decrease in the absorbance) matches with the CMC of the detergent ( $\sim 25 \text{ mM}$ ), which corresponds to a molar ratio of lipid to detergent of 1:6. For the detergents with the lower CMC's, the liposomes start to destabilize when the molar ratio of lipid to detergent is about 1:1. The relative high amounts of octyl glucoside necessary to destabilize the liposomes might also be a factor that negatively effects the functional reconstitution.

<sup>4</sup> J. Knol, G. Sulter, and B. Poolman, manuscript in preparation.

Although the structures formed by the lipid/detergent mixtures are hard to predict, the changes in absorbance offer a good diagnostic parameter for optimizing the reconstitution conditions. For  $C_{12}E_8$ , *n*-dodecyl- $\beta$ -D-maltoside and to lesser extent Triton X-100, the highest activities correspond with the maximal  $A_{540}$  value, presumably reflecting the saturation of the liposomes with detergent and the transition from the lamellar to the mixed micellar form (onset of solubilization). We speculate that at this point the LacS protein is inserted unidirectionally into the lipid bilayer by fusion of detergent-saturated liposomes and protein/detergent micelles. This fusion/insertion is improved by mixing the liposomes and protein at 20 °C rather than 4 °C (data not shown), which might be related to the increased fluidity of the lipid bilayer at higher temperatures and/or a change in the micellar molecular weight (Hjelmeland, 1980). Also, the rate of detergent removal is critical, as fast removal of the detergent decreased the transport activity of the proteoliposomes. A low Bio-Bead concentration is particularly important in the first step of the reconstitution in order to avoid loss of phospholipids and to allow the protein to insert into the detergent destabilized membrane. Once the protein is inserted into the bilayer, the Bio-Bead concentration is less critical but needs to be sufficient to remove the residual detergent molecules.

The advantage of reconstituting membrane proteins into detergent destabilized liposomes might be that one has a better control of the incorporation of the protein into the bilayer and that the protein is faced with lower detergent concentrations. In the case of  $H^+$ -ATPase,  $Ca^{2+}$ -ATPase, and other proteins (Richard *et al.*, 1990; Levy *et al.*, 1992; Rigaud and Pitard, 1995), it has been observed that a more uniform orientation is obtained when the proteins are reconstituted into preformed liposomes (Eytan, 1982). The large hydrophilic domains of these enzymes may prevent a scrambled orientation, which is usually observed when proteins are reconstituted from a suspension of mixed micelles (Rigaud and Pitard, 1995). A similar situation may hold for the LacS protein, which has a hydrophilic domain of about 180 amino acids at the COOH terminus. It is possible that this hydrophilic IIA domain forms an "anchor" that prevents the protein inserting randomly.

The kinetics of counterflow and of  $\Delta p$ -driven lactose uptake yield the same apparent affinity constants, *i.e.* approximately 0.17 mM. This is surprising since previous studies in *E. coli* indicated a  $K_m^{app}$  for  $\Delta p$ -driven lactose uptake of 0.8 mM, whereas the  $K_m^{app}$  for non-equilibrium exchange (equivalent to the counterflow activity measured in this study) was estimated to be 10 mM (Poolman *et al.*, 1992, 1995a). It should be stressed that the apparent affinity constants in previous determinations reflect the outside conformation of the protein whereas the present data correspond with the inside conformation of the LacS protein.

The higher  $V_{max}$  of the counterflow reaction as compared to the  $\Delta p$ -driven uptake reaction is in agreement with the observation that *S. thermophilus* transports lactose by exchange for galactose, a product of the lactose metabolism. The  $V_{max}$  of 6000 nmol/mg  $\times$  min reflects a turnover of  $\sim 7 s^{-1}$ , when it is

assumed that all the molecules are reconstituted functionally. With an expression level of LacS in wild type *S. thermophilus* of 1–2% of total cell protein, the turnover number of  $7 s^{-1}$  reflects an uptake rate of 60–120 nmol of lactose/min  $\times$  mg of total cell protein, which is similar to the *in vivo* lactose utilization (glycolysis) rate.

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