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Mechanism of Maltose Uptake and Glucose Excretion in *Lactobacillus sanfrancisco*

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Lactobacillus sanfrancisco **LTH 2581 can use only glucose and maltose as sources of metabolic energy. In maltose-metabolizing cells of *L. sanfrancisco*, approximately half of the internally generated glucose appears in the medium. The mechanisms of maltose (and glucose) uptake and glucose excretion have been investigated in cells and in membrane vesicles of *L. sanfrancisco* in which beef heart cytochrome *c* oxidase had been incorporated as a proton-motive-force-generating system. In the presence of ascorbate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD), and cytochrome *c*, the hybrid membranes facilitated maltose uptake against a concentration gradient, but accumulation of glucose could not be detected. Similarly, in intact cells of *L. sanfrancisco*, the nonmetabolizable glucose analog α-methylglucoside was taken up only to the equilibration level. Selective dissipation of the components of the proton and sodium motive force in the hybrid membranes indicated that maltose is transported by a proton symport mechanism. Internal [^{14}C]maltose could be chased with external unlabeled maltose (homologous exchange), but heterologous maltose/glucose exchange could not be detected. Membrane vesicles of *L. sanfrancisco* also catalyzed glucose efflux and homologous glucose exchange. These activities could not be detected in membrane vesicles of glucose-grown cells. The results indicate that maltose-grown cells of *L. sanfrancisco* express a maltose-H^+ symport and glucose uniport system. When maltose is the substrate, the formation of intracellular glucose can be more rapid than the subsequent metabolism, which leads to excretion of glucose via the uniport system.**

Transport of sugars in lactic acid bacteria can occur via the following mechanisms (19): (i) primary transport systems, which catalyze the translocation of sugars across the membrane at the expense of chemical energy, usually ATP; (ii) secondary transport systems, which use the (electro)chemical gradient of one solute (e.g., Na^+ or H^+) to drive the transport of the sugar (the sugar exchange systems also belong to this class of transport mechanisms); and (iii) phosphoenolpyruvate: sugar phosphotransferase systems, which phosphorylate the sugar in the translocation process.

In fermentative bacteria, an electrochemical proton gradient (proton motive force) can be generated by proton extrusion via F_0F_1-ATPase or by electrogenic secondary transport processes (16, 18, 19). Most frequently, secondary transport systems catalyze symport of solutes with protons or sodium ions (18, 19). The proton or sodium motive force is then used to drive solute transport. However, some secondary transport proteins catalyze exclusively an antiport. Examples of this class of transport are the systems which couple the uptake of precursor molecules to the excretion of product (precursor/product antiport) (16, 18). The driving forces for these processes are supplied by the (electro)chemical gradients of both precursor and product. An example of such an antiport system is the arginine/ornithine antiporter which has been detected in several bacteria (6, 17). It should be noted that most secondary transport systems, catalyzing solute-H^+ (or Na^+) symport, are able to mediate (heterologous) exchange of precursors with structurally related products. An example is the lactose transport system of *Streptococcus thermophilus*, which catalyzes efficient lactose/galactose exchange in addition to sugar-H^+ symport (9, 16, 20).

Lactobacillus sanfrancisco strains are the main organisms in sourdough starter preparations (1, 11, 23, 27). The prevailing sugar in sourdough is maltose, which is formed during degradation of starch by α-amylase. The metabolism of maltose in cells of *L. sanfrancisco* is initiated by cleavage of maltose to glucose and glucose-1-phosphate via a maltose phosphorylase (24). Glucose-1-phosphate and part of the free glucose are metabolized, whereas approximately half of the generated glucose is released into the external medium (25; this study). Since *L. sanfrancisco* is able to metabolize glucose, it was of interest to study the mechanism of maltose uptake and glucose excretion. For this purpose, the mechanisms of sugar transport in *L. sanfrancisco* were analyzed in membrane vesicles which were fused with cytochrome *c* oxidase-containing liposomes.

MATERIALS AND METHODS

Culture conditions and preparation of cells. *L. sanfrancisco* LTH 2581 was grown in MRS medium (3) supplemented with maltose or glucose (20 g/liter), cysteine-HCl (0.5 g/liter), and sodium acetate (5 g/liter) at 30°C at a constant pH of 5.8. Exponentially growing cells were harvested by centrifugation at an A_{660} of 2.0 to 2.5, washed twice with 100 mM KP_i (pH 7.0), and resuspended in a small volume of this buffer.

Glucose excretion by cells of *L. sanfrancisco*. Cells of *L. sanfrancisco* were starved for approximately 30 min by being washed with sugar-free sterile buffer. To start the experiment, the cells were suspended to a protein concentration of 2.4 mg/ml in sterile KP_i (100 mM, pH 6.1) supplemented with 2 mM maltose. Samples (0.5 ml) were taken at given time

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intervals and filtered through glass fiber filters (0.9-μm-pore size; Sigma). The filtrates were analyzed for glucose by the glucose oxidase-peroxidase method (GOD-Perid; Boehringer, Mannheim, Germany). Maltose was detected after enzymatic cleavage to glucose with α-glucosidase (final concentration, 16 U/ml; Boehringer) with the same test kit.

Preparation of membrane vesicles, liposomes, and hybrid membranes. Membrane vesicles of *L. sanfrancisco* were prepared as described by Otto et al. (15), using 350 mg of egg lysozyme per liter of culture and 0.3 mg of deoxyribonuclease and ribonuclease per ml (final concentrations). Preparation of cytochrome *c* oxidase-containing liposomes and fusion of the proteoliposomes with membrane vesicles were done as described by Driessen et al. (4, 5). Liposomes were prepared by using egg-phosphatidylcholine and purified *Escherichia coli* phospholipids (6398; Sigma) in a ratio of 1:3 (10).

Transport studies. (i) Intact cells. For the transport assays, concentrated cell suspensions were diluted in 100 mM KP_i (pH 6.1), supplemented with 2 mM $MgSO_4$. Experiments were initiated by adding L-[U-^{14}C]maltose, D-[U-^{14}C]glucose, or [α-U-^{14}C]methylglucoside. At given time intervals, samples (0.12 to 0.15 ml) were taken, diluted with 2 ml of ice-cold 0.1 M LiCl, and filtered through 0.45 μm cellulose-nitrate filters. Filters were washed with 2 ml of ice-cold 0.1 M LiCl, and radioactivity on the filters was measured by liquid scintillation spectrometry.

(ii) Hybrid membranes. Membrane vesicles of *L. sanfrancisco* fused with cytochrome *c* oxidase-containing liposomes were suspended to a final protein concentration of 0.25 mg/ml in 2.0 ml of CKC buffer (30 mM citrate, 30 mM K_2HPO_4, 30 mM CHES) at the appropriate pH (adjusted with KOH). The suspension was supplemented with 20 μM cytochrome *c* and 200 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). The electrochemical proton gradient was generated upon the addition of 10 mM ascorbate-KOH. During incubation, the mixture was aerated with water-saturated oxygen. After 1 min of energization, [^{14}C]maltose was added, and uptake was assayed as described above. To determine the kinetic parameters of transport, initial rates of maltose uptake were measured for up to 20 s. The intravesicular volume of the hybrid membranes was estimated from the equilibration of [^{14}C]methyl-β-D-thiogalactoside (final concentration, 1.1 mM) across the membrane over a period of 120 min.

(iii) Membrane vesicles of *L. sanfrancisco*. For efflux and exchange experiments, concentrated membrane vesicles of *L. sanfrancisco* were loaded with the appropriate concentration of [^{14}C]glucose by sonication for 20 s (bath sonicator). Loaded membrane vesicles were diluted 20-fold in buffer with and without maltose or glucose. Transport was stopped at different time intervals as described above.

Determination of the membrane potential. The membrane potential (ΔΨ, inside negative relative to the outside) was determined from the distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) across the membrane by using a TPP^+-selective electrode (8, 22). The membrane potential was calculated from the steady-state level of TPP^+ accumulation by using the Nernst equation. Correction for concentration-dependent binding of TPP^+ to the membrane was made according to the model described by Lolkema et al. (12).

Determination of the transmembrane pH gradient. The transmembrane pH gradient (ΔpH) was measured with the fluorescent membrane-impermeable dye 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine) as described previously (2).

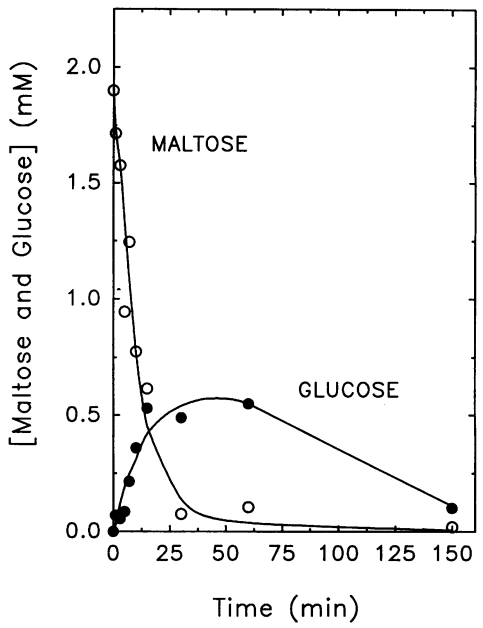

FIG. 1. Maltose utilization and release of glucose by resting cells of *L. sanfrancisco*. Cells were grown in MRS supplemented with maltose. Energy-starved cells were diluted to a final protein concentration of 2.4 mg/ml in 100 mM KP_i (pH 6.1) containing 2 mM maltose. Maltose utilization and glucose excretion were analyzed at 30°C, and sugar concentrations were determined as described in Materials and Methods.

Protein determination. Protein was measured by the method of Lowry et al. (13) in the presence of sodium dodecyl sulfate (7) with bovine serum albumin as the standard.

Materials. The radiolabeled sugars [U-^{14}C]maltose (630 mCi/mmol), D-[U-^{14}C]glucose (273 mCi/mmol), [α-U-^{14}C]methylglucoside (197 mCi/mmol), and [U-^{14}C]methyl-β-D-thiogalactoside (56 mCi/mmol) were obtained from Amersham (Amersham, United Kingdom). Pyranine was obtained from Eastman Kodak Co., Rochester, N.Y. All other chemicals were reagent grade and were obtained from commercial sources.

RESULTS

Glucose excretion by *L. sanfrancisco* cells. Resting cells of *L. sanfrancisco*, grown in the presence of maltose, were incubated in maltose-containing buffer, and maltose utilization and glucose production were monitored. The maltose was rapidly consumed, and most of the maltose was used after 25 min (Fig. 1). Interestingly, approximately half of the glucose generated was excreted into the external medium within the first 15 min. The glucose levels remained constant until all the maltose was consumed, after which the excreted glucose was consumed.

Sugar transport in whole cells. Cells of *L. sanfrancisco* grown on maltose take up [^{14}C]maltose and [^{14}C]glucose at a high rate (Fig. 2). Since maltose and glucose are rapidly metabolized, the accumulated radioactivity will be present mainly in metabolites formed from the sugars. Therefore, it is not possible to establish whether the sugars are accumulated against their concentration gradients. Transport of both maltose and glucose was not affected by a fourfold excess of the other sugar, suggesting that both sugars enter the cell via separate transport systems (data not shown). The glucose analog α-methylglucoside was taken up only to the equilibra-

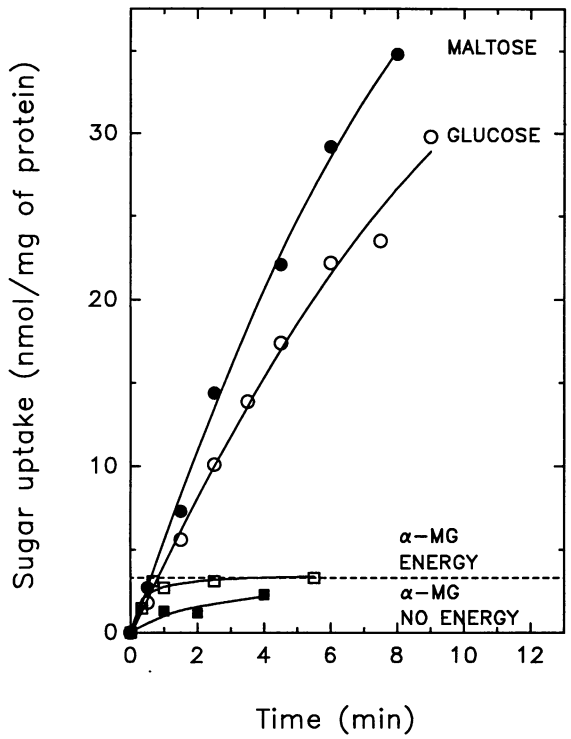

FIG. 2. Sugar uptake in whole cells of *L. sanfrancisco*. Cells of *L. sanfrancisco* grown on maltose as a carbon and energy source were washed and resuspended to a protein concentration of 2.0 mg/ml in 100 mM KP_i containing 2 mM $MgSO_4$ (pH 6.1). L-[U-^{14}C]maltose (0.2 mCi/ml; 2.6 mM, final concentration; closed circles) or D-[U-^{14}C]glucose (0.8 mCi/ml; 2.6 mM, final concentration; open circles) was added at zero time. Uptake of [α-U-^{14}C]methylglucoside (α-MG; 1.2 mCi/ml; 0.75 mM, final concentration; squares) was measured in KP_i (100 mM) at pH 7.5 following 3 min of preincubation with (open squares) or without (closed squares) 20 mM maltose. Sugar uptake was assayed at 30°C.

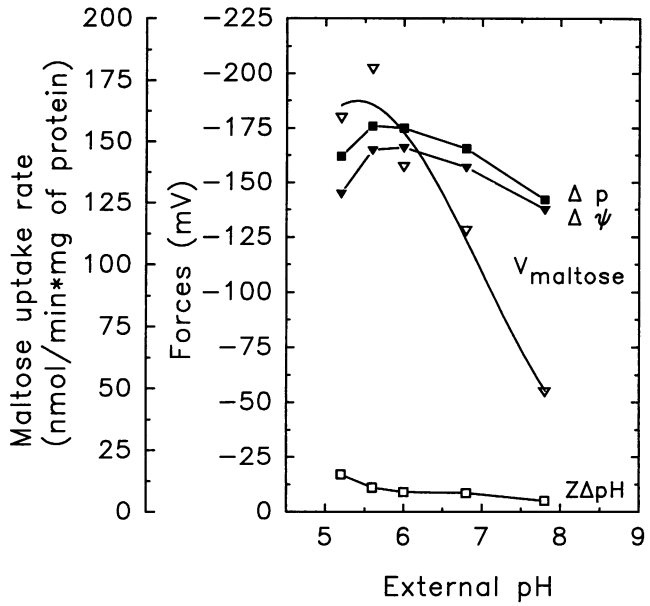

FIG. 3. Effect of external pH on ΔΨ, ΔpH, and Δ*p* and initial rate of maltose uptake in cytochrome *c* oxidase-containing hybrid membranes prepared from maltose-grown *L. sanfrancisco* cells. The steady-state values of ΔΨ, ΔpH, and Δ*p* and the rate of maltose uptake were determined after 5 min of energization in the presence of ascorbate-TMPD-cytochrome *c*. Initial rates of maltose transport were estimated from the linear parts of the curves, i.e., from the uptake for up to 20 s. Uptake of L-[^{14}C]maltose was assayed at a final concentration of 1.2 mM. The final protein concentrations were 9.1, 0.8, and 77 μg/ml for the determination of ΔΨ, ΔpH, and initial uptake rates, respectively.

tion level, even in the presence of maltose as a source of metabolic energy (Fig. 2). The uptake of α-methylglucoside was completely inhibited by an excess of unlabeled glucose (data not shown).

Generation of a proton motive force and sugar transport in hybrid membrane vesicles. To avoid the interference of sugar metabolism, transport was studied further in membrane vesicles of *L. sanfrancisco* fused with liposomes containing beef heart mitochondrial cytochrome *c* oxidase as a proton-motive-force-generating system (hybrid membrane vesicles). In the presence of the electron donor system, ascorbate-TMPD-cytochrome *c*, a proton motive force (Δ*p*) inside negative and alkaline relative to the outside, was generated in the hybrid membrane vesicles. The magnitude and composition of the Δ*p* were determined at different pH values. The hybrid membrane vesicles of *L. sanfrancisco* maintained a relatively constant Δ*p* of approximately −160 mV in the pH range of 5.2 to 7.8, with an optimum around pH 6.0 (Fig. 3). The ΔpH was a minor component of Δ*p* at every pH value tested.

Maltose was rapidly taken up in response to a proton motive force by hybrid membranes prepared from maltose-grown cells (Fig. 3). The intravesicular volume of the hybrid membrane vesicles was estimated to be 27 μl/mg of protein, and by using this value, a 20-fold accumulation of maltose could be calculated after 10 min of uptake at pH 5.6 (data not shown). Since the net uptake of maltose continued for more than 10 to 15 min (Fig. 4 and 5), for technical reasons it was not possible to reach steady-state accumulation levels (under the assay conditions, Δ*p* decreases after 10 to 15 min). Similar rates of uptake and levels of maltose accumulation were observed with hybrid membranes prepared from membrane vesicles of glucose-grown cells. Since *L. sanfrancisco* is able to grow only on maltose and glucose as the carbon and energy sources, it can be concluded that the maltose transport system is expressed constitutively. In hybrid membranes prepared from membrane vesicles of both maltose- and glucose-grown cells, accumulation of glucose could not be detected upon the addition of the electron donor system ascorbate-TMPD-cytochrome *c* (data not shown).

Roles of ΔΨ, ΔpH, and Δ*p*Na in maltose transport. To analyze whether the membrane potential (ΔΨ), pH gradient (ΔpH), and/or sodium gradient (Δ*p*Na) were able to drive maltose uptake, the Δ*p* generated by ascorbate-TMPD-cytochrome *c* oxidation in the hybrid membranes was selectively manipulated with ionophores in the presence or absence of Na^+. Valinomycin, a ionophore which allows the selective influx of K^+ and causes the dissipation of the membrane potential, gave a strong inhibition of maltose uptake (Fig. 4A). Dissipation of the ΔpH with nigericin (a potassium/proton exchanger) did not significantly affect maltose transport (Fig. 4A), a result which is consistent with the small ΔpH in the hybrid membranes (Fig. 3). Moreover, dissipation of the ΔpH by nigericin may result in a compensatory increase in ΔΨ such that the total Δ*p* is largely unaffected. By abolishing both the ΔΨ and the ΔpH with valinomycin plus nigericin, the uptake of maltose was inhibited completely and the efflux of accumulated

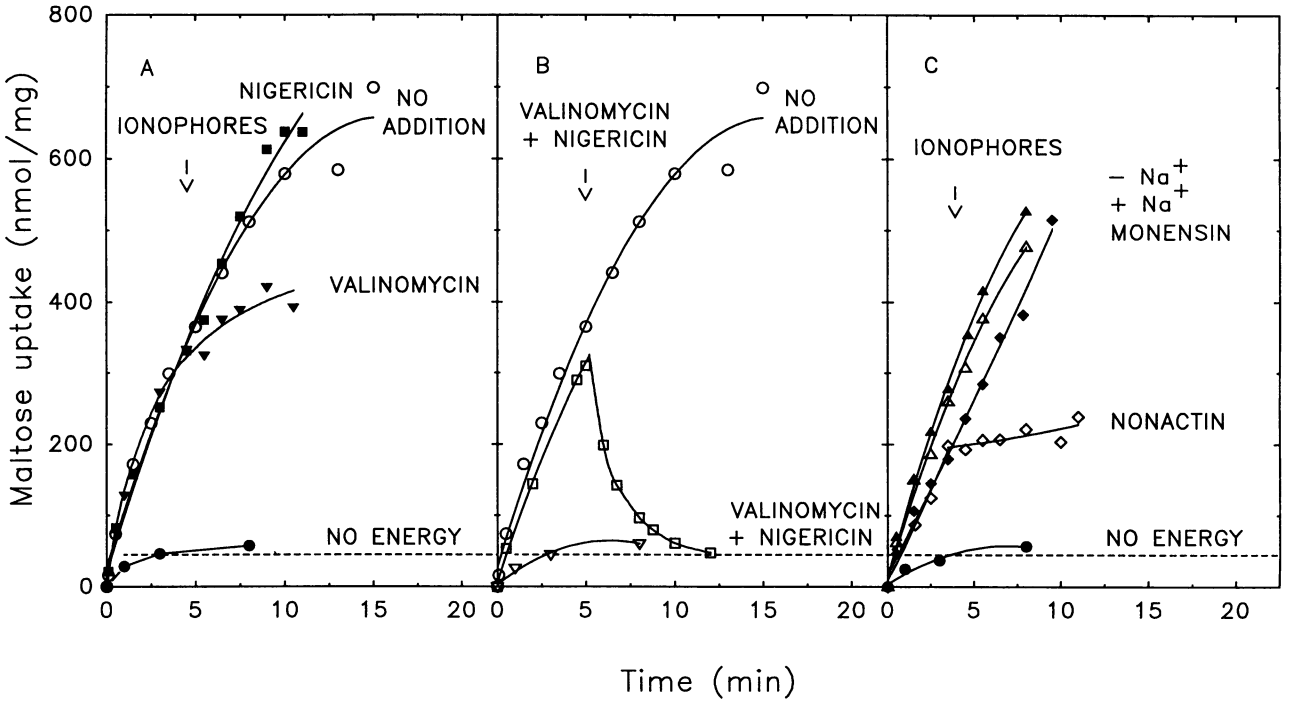

FIG. 4. Effect of ionophores on maltose uptake in cytochrome *c*-oxidase-containing hybrid membranes of *L. sanfrancisco*. The rates of uptake of L-[^{14}C]maltose (1.2 mM, final concentration) were determined at 30°C in CKC buffer (pH 5.6) at a final protein concentration of 0.18 mg/ml unless stated otherwise. The electron donor system was composed of ascorbate-TMPD-cytochrome *c*. The equilibration level is indicated by a broken line; the addition of ionophores is indicated by arrows. (A) Control membranes were incubated with L-[^{14}C]maltose in the absence of the electron donor (closed circles). Maltose uptake in the presence of electron donor systems is represented by the open circles. Nigericin (0.25 μM, final concentration; closed squares) or valinomycin (0.5 μM, final concentration; closed triangles) was added 5 min after the addition of maltose. (B) Maltose uptake in the presence of the electron donor system and with valinomycin plus nigericin (added at zero time) is represented by the open triangles. The effects of valinomycin and nigericin on maltose transport after 5 min of uptake in the presence of electron donor are indicated by the open squares. (C) Effect of sodium on maltose uptake. Experiments were performed in plastic tubes with potassium phosphate low in contaminating sodium (Merck). Closed triangles represent maltose uptake without the addition of Na^+; open triangles represent uptake in the presence of 10 mM NaCl. Monensin (0.25 μM, final concentration; closed diamonds) or nonactin (10 μM, final concentration; open diamonds) was added 4 min after maltose uptake was initiated in buffer supplemented with 10 mM NaCl.

maltose was observed (Fig. 4B). These observations indicate that maltose is translocated in symport with a proton(s). The possible involvement of a sodium gradient in the uptake of maltose was also studied. The rate of uptake of maltose in the presence of less than 10 μM Na^+ was essentially the same as that in the presence of 10 mM Na^+ (Fig. 4C). Conversion of the ΔpH into a Δ*p*Na by the Na^+/H^+ exchanger monensin also did not enhance the maltose uptake rate. However, dissipation of both the membrane potential and the Na^+ gradient by the sodium ionophore nonactin strongly decreased maltose accumulation (Fig. 4C). These observations argue against a role of Na^+ in the maltose transport process.

Effect of pH and substrate concentration on maltose transport activity. To determine the effect of pH on maltose transport activity, initial uptake rates were determined in cytochrome *c* oxidase-containing hybrid membranes of maltose-grown *L. sanfrancisco* cells which had been equilibrated at different pH values. The maltose uptake rate was maximal at low external pHs (5.2 and 5.6) and decreased with increasing pH (Fig. 3). Since the ΔpH was relatively small at all pH values tested, the internal pH also dropped with decreasing pH of the buffer, i.e., from pH 7.9 to 5.4, in the pH range of 7.8 to 5.2. Kinetic studies of maltose uptake in the hybrid membranes were done with CKC buffer (pH 5.6). Initial rates of maltose uptake were determined over a wide concentration range (55 μM to 20 mM), and the kinetic data were transformed into Eadie-Hofstee plots. From a single straight line which resulted, an apparent Michaelis-Menten constant (K_m^{App}) of 2.9 mM for maltose uptake and a V_{max} of 415 nmol/min/mg of protein could be calculated.

Sugar exchange and efflux. The ability of the maltose transport system to catalyze exchange was studied by adding an excess of unlabeled maltose or glucose to [^{14}C]maltose-loaded cytochrome *c* oxidase-containing hybrid membranes (Fig. 5). Internal [^{14}C]maltose could be released by an excess of unlabeled maltose (homologous exchange) but not by an excess of glucose (heterologous exchange). These results, together with the observation that maltose-metabolizing cells excrete glucose (Fig. 1), suggest that a separate efflux system exists for glucose. The presence of such a system was investigated by diluting membrane vesicles prepared from maltose-grown cells and loaded with [^{14}C]glucose into a buffer containing 10 mM unlabeled glucose or maltose or no sugar (Fig. 6). Rapid exit of [^{14}C]glucose was observed in the presence of external glucose, whereas release of [^{14}C]glucose was much slower in the absence of sugar or in the presence of maltose. Membrane vesicles from glucose-grown cells, which were loaded with [^{14}C]glucose, did not catalyze efflux or the exchange of glucose (Fig. 6). These experiments indicate that glucose efflux in maltose-grown cells is catalyzed by a transport system which is induced during

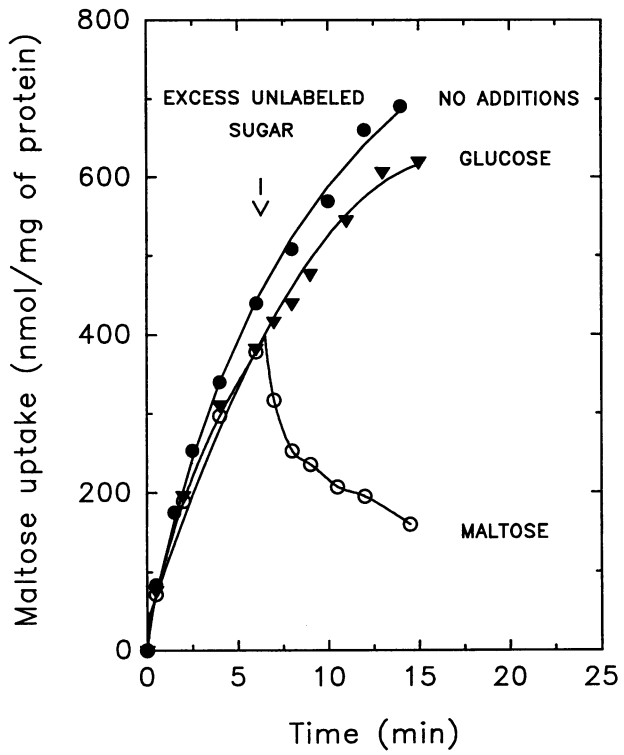

FIG. 5. Effect of excess of maltose and glucose on accumulated [^{14}C]maltose in hybrid membranes of *L. sanfrancisco*. The rates of uptake of L-[^{14}C]maltose (1.2 mM, final concentration) were determined at 30°C in CKC buffer (pH 5.6) at a final protein concentration of 0.21 mg/ml and with ascorbate-TMPD-cytochrome *c* as the electron donor system. No further additions were made (closed circles), or a 100-fold excess of unlabeled maltose (open circles) or glucose (triangles) was added after 6.5 min of uptake.

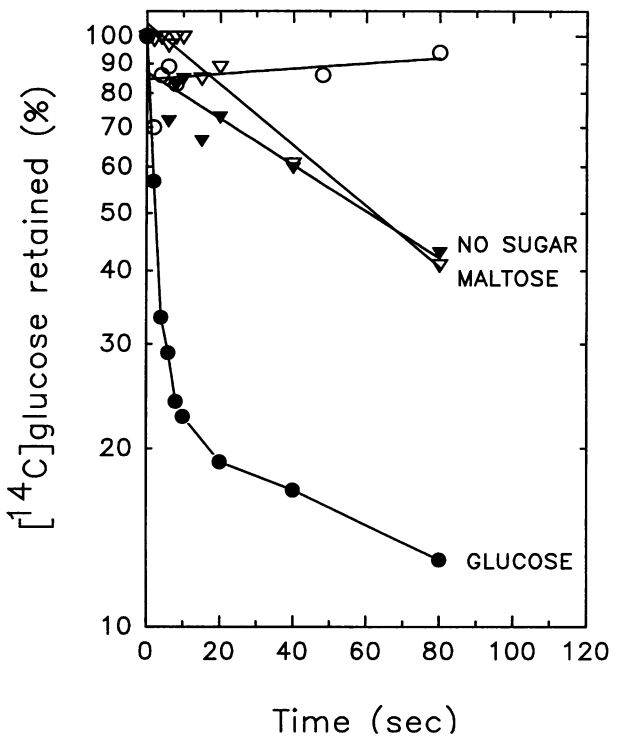

FIG. 6. Efflux and exchange of glucose in membrane vesicles of *L. sanfrancisco*. Cytochrome *c* oxidase-containing hybrid membrane vesicles from maltose-grown cells were loaded by sonication with D-[^{14}C]glucose to a final concentration of 5.2 mM. Experiments were performed at 0°C upon 20-fold dilution of the membrane vesicles into CKC buffer (pH 5.75) containing 10 mM glucose (closed circles), 10 mM maltose (open triangles), or no sugar (closed triangles). The final protein concentration of the membranes was 0.6 mg/ml. Membrane vesicles from glucose-grown cells were loaded in a similar way and diluted to a final concentration of 0.4 mg/ml in CKC buffer without sugar (open circles).

growth on maltose. This system can mediate homologous glucose/glucose exchange but not maltose/glucose exchange.

DISCUSSION

Resting cells of *L. sanfrancisco* that are cultured on maltose metabolize maltose rapidly but excrete approximately half of the internally generated glucose into the medium. After depletion of maltose, the excreted glucose is taken up and metabolized by the cells. The mechanisms responsible for the uptake of maltose and excretion of glucose have been investigated in this study. Maltose transport has been reported to occur via a PTS system in several streptococcus species (14, 28) and via a proton-motive-force-dependent system in *Bacillus licheniformis* and *Bacillus subtilis* (26). The results presented in this paper clearly demonstrate that maltose transport in *L. sanfrancisco* occurs by a secondary transport system and is driven by the proton motive force. Maltose accumulation could be demonstrated in cytochrome *c* oxidase-containing hybrid membranes of both maltose- and glucose-grown cells of *L. sanfrancisco*, indicating that the maltose carrier protein(s) is constitutively expressed. The activity of the maltose carrier is highly dependent on pH (under conditions that Δp is nearly constant) with optimum activity at pH 5.6 to 5.2. The relatively acidic pH at which the activity is highest matches well with the pH of the environment in which *L. sanfrancisco* normally ferments; rye sourdough has an initial pH of about 5.6, which decreases to about 4.3 during fermentation. It should be noted that the ΔpH in the hybrid membranes is rather low and, most likely, lower than in maltose-metabolizing cells, which may not fully justify a direct comparison of the in vitro and in vivo data. Kinetic analysis of Δp-driven maltose uptake revealed that the maltose carrier of *L. sanfrancisco* has a relatively low affinity for its substrate (K_m^{App} of 2.9 mM) but a high V_{max}.

To account for the release of glucose during maltose utilization, experiments were set up to discriminate between glucose exit via the maltose carrier (possible maltose/glucose exchange) or via a separate efflux system for glucose. Our results indicate that maltose-grown cells of *L. sanfrancisco* express a glucose efflux (and exchange) activity which is not observed in glucose-grown cells. In contrast to maltose, no accumulation of glucose is observed in response to a Δp in cytochrome *c* oxidase-containing hybrid membrane vesicles prepared from maltose- or glucose-grown cells. The finding that intact cells of *L. sanfrancisco* grown on maltose or glucose transport the nonmetabolizable glucose analog α-methylglucoside only up to the equilibration level (i.e., the internal concentration becomes equal to the external one) suggests the presence of a uniport system. The α-methylglucoside uptake system most likely corresponds to the system that catalyzes glucose efflux (and exchange) in the membrane vesicles. The stimulation of α-methylglucoside uptake in the presence of a metabolizable sugar (maltose; Fig. 2) is most likely due to a

change (increase) in the internal pH, which activates the glucose carrier protein. Taken together, our results indicate that *L. sanfrancisco* expresses a maltose-inducible glucose uniport system that is most likely responsible for the excretion of excess of glucose during growth on maltose. The mechanism of glucose transport by glucose-grown cells has not been analyzed in detail, but our results argue against a secondary active transport system (the accumulation of glucose in response to a proton or sodium motive force was not observed).

The reason for glucose excretion is not immediately clear. Internally generated glucose has to be phosphorylated by a hexokinase for further degradation. However, the activity of this enzyme in maltose-grown cells of *L. sanfrancisco* ATCC 27651 is rather low (0.03 U/ml at a final glucose concentration of 30 mM, for cells harvested in the mid-logarithmic phase of growth [24]). Higher activities are found under the same conditions in glucose-grown cells (0.15 U/ml), indicating that expression of hexokinase is induced by glucose (a similar situation is most likely true for *L. sanfrancisco* LTH 2581, used in this study). Uptake of maltose at a high rate, followed by hydrolysis to glucose and glucose-1-phosphate by the maltose phosphorylase, could lead to the production of glucose at a rate that exceeds the rate of conversion of glucose to glucose-6-phosphate by hexokinase. To avoid the generation of excessive concentrations of free glucose inside the cell, *L. sanfrancisco* releases part of the glucose via the uniport mechanism. Excreted glucose is utilized after maltose has been depleted from the medium.

ACKNOWLEDGMENT

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