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Proton-Linked D-Xylose Transport in *Escherichia coli*

VERONICA M. S. LAM,† KATIE R. DARUWALLA,‡ PETER J. F. HENDERSON,* AND MAURICE C. JONES-MORTIMER

Department of Biochemistry, University of Cambridge, Cambridge, CB2 1QW, United Kingdom

The addition of xylose to energy-depleted cells of *Escherichia coli* elicited an alkaline pH change which failed to appear in the presence of uncoupling agents. Accumulation of [^{14}C]xylose by energy-replete cells was also inhibited by uncoupling agents, but not by fluoride or arsenate. Subcellular vesicles of *E. coli* accumulated [^{14}C]xylose provided that ascorbate plus phenazine methosulfate were present for respiration, and this accumulation was inhibited by uncoupling agents or valinomycin. Therefore, the transport of xylose into *E. coli* appears to be energized by a proton-motive force, rather than by a phosphotransferase or directly energized mechanism. Its specificity for xylose as inducer and substrate and the genetic location of a xylose-H^+ transport-negative mutation near *mtl* showed that the xylose-H^+ system is distinct from other proton-linked sugar transport systems of *E. coli*.

The transport of lactose, D-galactose, or L-arabinose across the cell membrane of *Escherichia coli* is linked to the movement of protons (14, 39). This supports the chemiosmotic mechanism proposed by Mitchell (27, 28) and West and Mitchell (40), whereby the transport process is energized by a trans-membrane electrochemical gradient of protons. Such a mechanism is distinct from the vectorial phosphorylation of sugars by phosphoenolpyruvate (reviewed in 12, 30), or the direct energization of transport by a product of glycolysis, possibly ATP (4, 5) or acetyl phosphate (18). Of the two separate systems for galactose transport in *E. coli*, one is apparently directly energized and the other is energized by the proton-motive force (16); these two mechanisms also operate for the two arabinose transport systems in *E. coli* (P. J. F. Henderson and K. R. Daruwalla, Abstr. 11th FEBS Meet., Copenhagen, abstr. no. B7-2 375 4). The experimental criteria applied to distinguish the mechanisms of energization for galactose or arabinose transport are extended to the xylose transport system in this study.

The existence of a transport system for xylose in *E. coli* has been reported previously (1, 8). The genes coding for transport (*xylT*), xylose isomerase (*xylA*), and xylulokinase (*xylB*) are probably regulated as an operon. They map at min 79 near the *mtl* marker on the linkage map (3, 8, 36, 37).

† Permanent address: Department of Biochemistry, University of Hong Kong, Hong Kong.

‡ Present address: National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD.

MATERIALS AND METHODS

Organisms. The wild-type *E. coli* strain VL17 used in these experiments is an Fda$^+$ and, therefore, xylose-positive transductant of strain JM559 (P.J.F.H., R. A. Giddens, and M.C. J-M., 1977, Suppl. Publication SUP 50074, obtainable from the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K.). Strain JM559 is Hfr Cavalli *fda*(Ts) Δ(*his-gnd*). The xylose-negative mutant, strain VL18, was obtained from strain JM559 in two stages. First, a spontaneous xylose-resistant mutant, strain VL1, was isolated on glycerol at 40°C (an adaptation of the technique of Jones-Mortimer and Kornberg [20]), and this mutant was then transduced to Fda$^+$ to obtain strain VL18. Selection for Fda$^+$ was on fructose as sole carbon source at 40°C; bacteriophage P1 grown on strain K10 was used as the donor.

Preparation of cells for transport measurements. *E. coli* strains were grown in salts medium (16) supplemented with 10 mM xylose and 20 mM glycerol at 30°C in a Gallenkamp orbital incubator operating at 250 rpm. Cultures of absorbance A_{680} = 0.8-1.0 were harvested by centrifugation, suspended in an equal volume of 150 mM KCl-5 mM glycylglycine (pH 6.8)-1 mM mercaptoethanol, and then reincubated for 1 h. These energy-depleted cells were harvested and washed once with an equal volume of 150 mM KCl-2 mM glycylglycine (pH 6.8) and finally resuspended in the same buffer to A_{680} = 45-55 (30 to 38 mg of cell [dry mass] per ml).

The final wash and resuspension were carried out in 150 mM KCl-5 mM glycylglycine (pH 6.8) to A_{680} = 1.8-2.2 when preparing cells for measurements of radioisotope-labeled sugar transport. The use of 5 mM instead of 2 mM glycylglycine scarcely affected the sugar transport, but the increased buffering capacity prevented undesirable pH changes when metabolizable sugars were used.

Measurement of sugar-promoted pH changes.

Bacterial suspension equivalent to 12 mg (dry mass) was diluted into a total volume of 4.0 ml of de-aerated 150 mM KCl–2 mM glycylglycine (initial pH 6.8, temperature 20°C) contained in a glass cell very similar to that described by Henderson et al. (16). Additions of 14-μl 0.5 M de-aerated sugar solutions were made when the recording from the pH electrode immersed in the suspension indicated a low rate of drift, some 10 to 15 min after introduction of the bacterial suspension. The pH was 6.4 to 6.8. Deflections were converted to ΔH^+ by calibration with 3 μl or 5 μl of air-free standard 0.01 M NaOH. Further experimental details were described by Henderson et al. (16).

Measurements of energized sugar transport. A 0.5-ml sample of the cell suspension (A_{680} = 1.8–2.2) containing 10 mM glycerol was bubbled with air for three min at 20°C. Radioisotope-labeled sugar (25 nmol, 2.5–2.0 Ci/mol) was then added, and 0.2-ml samples were withdrawn into an Eppendorf pipette and discharged onto a Sartorius (SM 11306) or Oxoid (W25/45 UP) 0.45-μm-pore-size filter at 15 s and 2 min. Each sample was filtered by suction and washed twice with approximately 2 ml of suspension medium. The radioactivity retained on a filter was measured in 10 ml of scintillant containing toluene (80%), 2-methoxyethanol (20%), 2,5-diphenyloxazole (4 g/liter), 1,4-bis (5-phenoxazol-2-yl) benzene (0.2 g/liter). The labeled sugar (5 nmol) was also counted under identical conditions and used to convert counts in the experimental vials into nanomoles of sugar after correction for background radiation.

RESULTS

Xylose-promoted pH changes. A culture of VL17 grown on xylose plus glycerol was harvested and suspended under anaerobic conditions as described above. After a period of equilibration, the addition of xylose evoked an immediate alkaline pH change followed by a prolonged acidification (Fig. 1). The alkaline pH change is interpreted as an influx of protons (or the indistinguishable efflux of hydroxyl ions) that accompanies xylose transport. The subsequent acidification is almost certainly due to metabolism of the xylose to organic acids such as lactate, acetate, and succinate (14–16).

The appearance of the alkaline pH change was prevented by uncoupling agents such as tetrachlorosalicylanilide (TCS) (Fig. 1), 2,4-dinitrophenol, sodium azide, and carbonylcyanide *m*-chlorophenylhydrazone (CCCP). These compounds render the membrane permeable to protons (11, 26), and so would allow the proton carried with the xylose to pass back out through the membrane. Susceptibility to uncouplers implies that the alkaline pH change is due to a transport of protons, rather than binding or secretion of a metabolic product.

Xylose promoted a similar uncoupler-sensitive alkaline pH change, followed by acid secretion, with *E. coli* strain K10 grown on xylose plus glycerol (Table 1).

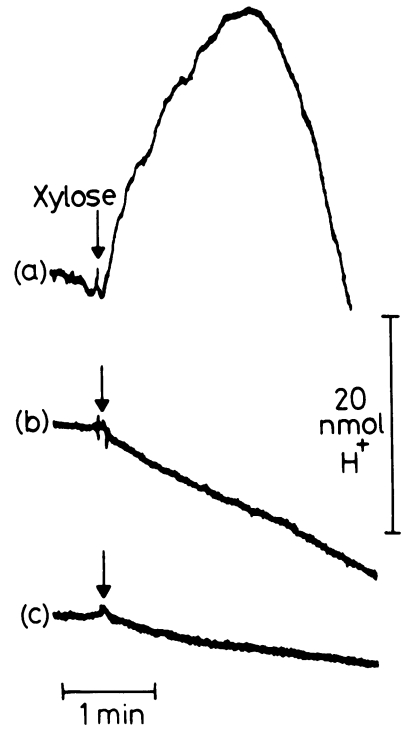

FIG. 1. *pH changes elicited by the addition of xylose to energy-depleted suspensions of E. coli. Strain VL17 (a, b) and strain VL18 (c) were grown on glycerol plus xylose. The cells were depleted and suspended as described in the text, and the pH was recorded after a period of equilibration. At the point indicated by the arrow, 7 μmol of xylose was added. Tetrachlorosalicylanilide (10 μM) was included in the suspension for experiment (b). An upward deflection indicates an alkaline pH change.*

Inducer specificity of the xylose-H^+ symport system. In various experiments *E. coli* strains VL17 and K10 were grown in the presence of xylose, ribose, arabinose, fucose (gratuitous inducer of the galactose transport systems GalP and MglP [34]), isopropyl-β-D-thiogalactoside (a gratuitous inducer of the lactose transport system LacY [19]), and glycerol. Xylose-H^+ activity only appeared when the organisms were grown in the presence of xylose (Table 1).

In each case the appropriate substrate of the induced proton symport system—LacY, GalP, AraE—was also added, to confirm that induction had occurred (see below). With cells induced for AraE, xylose promoted a slow alkaline pH change at about 10% of the rate achieved with the normal substrate arabinose (K.R.D., Ph.D. thesis, University of Cambridge, 1979). Arabi-

TABLE 1. *Inducibility of the xylose-H⁺ symport system*[a]

Strain	Carbon compounds in growth medium	Xylose-promoted H⁺ uptake (nmol)	Rate of H⁺ uptake (nmol min⁻¹)	H⁺ efflux
VL17	Glycerol + xylose	40.4[b]	18.3[b]	+
	Glycerol	0	0	–
	Arabinose	0	0	–
	Glycerol + fucose	0	0	–
K10	Glycerol + xylose	19.0[b]	13.1[b]	+
	Arabinose	S.a.d.[c]	4.5	–
VL18	Glycerol + xylose	0	0	–
	Arabinose	S.a.d.[c]	1.7	–
	Glycerol + xylose + isopropyl-β-D-thiogalactoside	0	0	–

[a] *E. coli* strains were grown in the presence of 20 mM glycerol, 10 mM xylose, 10 mM arabinose, 1 mM D-fucose, or 0.5 mM isopropyl-β-D-thiogalactoside. Xylose-promoted pH changes were measured as described in the text and in the legend to Fig. 1.

[b] Mean of four measurements.

[c] S.a.d., Slow alkaline drift.

nose did not promote an alkaline pH change with xylose-induced cells (Table 2). Hence, xylose is a poor substrate for the arabinose system (see also 29) but arabinose is not an inducer or substrate for the xylose system.

Substrate specificity of the xylose-H⁺ symport system. L-Arabinose, D-ribose, D-lyxose, xylitol, and D-fucose failed to promote pH changes in xylose-induced strains VL17 and K10, as shown in Table 2. Since arabinose and fucose elicit pH changes in *E. coli* strains induced for the arabinose-H⁺ symport system AraE (K.R.D. Ph.D. thesis) and since fucose is a substrate for the galactose-H⁺ symport system GalP (16), these results confirm the absence of AraE and GalP in the xylose-induced strains. Ribose uptake into *E. coli* probably does not utilize a proton-linked transport system (2, 6, 9, 41), and ribose failed to elicit a pH change with the xylose-induced cells (Table 2), so it is unlikely that a ribose transport system is involved in xylose transport (see also 8, 9). D-Lyxose and xylitol are not normal substrates for growth of *E. coli*, but they have been reported to be substrates for a xylose transport system in mutant strains (38, 42, 43). However, they did not elicit a pH change and so are presumably not substrates for the xylose-H⁺ system.

Methyl-β-D-thiogalactoside (TMG), a substrate of the LacY transport system, promoted an alkaline pH change in xylose-induced VL17 (Table 2). This is apparently an artifact due to the inadvertent selection of a derepressed lactose mutant as indicated by the relatively high TMG uptake into strain VL17 (see Table 4). Strain K10 did not take up TMG when induced with xylose (Table 4), nor did strain VL18 take up xylose when induced with isopropyl-β-D-thiogalactoside (data not shown).

Susceptibility of energized xylose transport to uncoupling agents and metabolic inhibitors. Cells of strain VL17 grown on glycerol plus xylose were harvested and suspended under aerobic conditions with glycerol as respiratory substrate. Radioisotope-labeled xylose was accumulated rapidly by these cells (Table 3). Accumulation was inhibited by uncoupling agents used at concentrations that render the bacterial membrane permeable to protons (Table 3); since CCCP and TCS were dissolved in ethanol, these results were compared with controls in which a slight inhibition was observed with an equivalent volume of solvent alone (Table 3). The sensitivity to uncouplers indicated that xylose was taken up by active transport energized by a transmembrane proton gradient and not by a phosphotransferase mechanism (11); had the transport been mediated by a phosphotransferase mechanism, a stimulation of transport would have been predicted (17, 24). Fluoride, which prevents phosphoenolpyruvate formation by the enolase reaction, actually enhanced xylose transport (Table 3).

Arsenate drastically decreases the intracellular concentration of ATP (24) and has been shown to inhibit the so-called "shockable" trans-

TABLE 2. *Sugar specificity for the xylose-H⁺ symport system*[a]

Strain	Sugar added	Effective H⁺ uptake (nmol)	Rate of H⁺ uptake (nmol min⁻¹)	H⁺ efflux
VL17	D-Xylose	40.4	18.3	+
	TMG	57.3	25.0	–
	L-Arabinose	0	0	–
	D-Fucose	0	0	–
	D-Ribose	0	0	–
K10	D-Xylose	19.0	13.1	+
	TMG	0	0	–
	D-Lyxose	0	0	–
	Xylitol	0	0	–

[a] The organisms were grown on 10 mM xylose plus 20 mM glycerol, harvested, and prepared for measuring pH changes as described in the text. Xylose (7 μmol) was added to 12 mg (dry mass) of bacteria for each experiment.

TABLE 3. *Effect of metabolic inhibitors on xylose transport*[a]

Addition	Concn	Activity (% of control)
None	—	100
96% Ethanol	2% (vol/vol)	80
CCCP	5 μM	51
	20 μM	6
2,4-Dinitrophenol	1 mM	31
	5 mM	7
TCS	5 μM	65
	20 μM	9
Sodium fluoride	30 mM	168
Sodium arsenate	5 mM	101
	50 mM	91

[a] Strain VL17 was grown on 20 mM glycerol plus 10 mM xylose. The final suspension of cells was exposed to 10 mM glycerol plus the indicated addition for 3 min and then the transport of radioisotope-labeled xylose was measured as described in the text. Control rate was 10.0 nmol/min per mg, or 7.9 nmol/min per mg in the presence of 2% (vol/vol) ethanol, the solvent for CCCP and TCS.

port systems (4, 5), possibly through lowering intracellular acetyl phosphate concentrations (18). Potassium arsenate (5 mM) reduced activity of the shockable MglP and AraF systems by 80% and 60% whereas the chemiosmotic LacY, GalP, and AraE systems were reduced by 40, 30, and 5% (K.R.D., Ph.D. thesis). Thus, sensitivity to arsenate discriminates reasonably well between the two energization mechanisms (see also 4–6, 33). In fact, xylose transport was unaffected by 5 mM arsenate and even 50 mM arsenate only reduced transport by 10% (Table 3).

These results imply that xylose transport is energized by a chemiosmotic mechanism and not by a phosphotransferase or directly energized mechanism.

Substrate and inducer specificity of energized xylose transport. The transport of xylose and related sugars into appropriately induced cultures of strain VL17 was measured. From the results in Table 4 it is apparent that arabinose, fucose, and galactose are not substrates for the xylose transport system. The corollary of this is that xylose uptake does not occur on the AraE, AraF, GalP, or MglP sugar transport systems, which is borne out by the absence of xylose transport when only the appropriate inducers of these other systems are present during growth (Table 4).

Table 4 also shows relatively high rates of TMG uptake into strain VL17 regardless of the inducer present, indicating the presence of a derepressed LacY system as discussed above. Although ribose transport activity was not measured, the absence of xylose transport in cells grown on ribose indicates that xylose is not a substrate for the ribose transport system (Table 4). Similarly, xylose does not appear to be a good substrate for glucose transport system(s) (Table 4). Uptake of glucose by the xylose transport system could not be examined because of the high endogenous rates of glucose transport in uninduced strains VL17 and K10. Furthermore, xylose was the only sugar that induced xylose transport (Table 4). These results correlate very well with the substrate and inducer specificity of the xylose-H^+ system and indicate that it is responsible for the energized transport of xylose.

Loss of xylose-H^+ activity and energized transport in a Xyl^- mutant. Strains VL1 and VL18, unable to grow on xylose, were isolated as described above. The genetic location of the xylose-negative lesion was established as follows. Bacteriophage P1 grown on strain VL1 was used to transduce strain JM1515 [*mtlA fda*(Ts) Δ(*his-gnd*) Δ(*gal-uvrB*) *ptsM ptsF srlA*]. Recombinants were selected on mannitol medium at 30°C and tested for their ability to utilize xylose as sole carbon source. Of 161 mannitol-positive colonies, 23% were xylose negative. Solomon and Lin (37) obtained 15% cotransduction between *xyl* and *mtl*. We therefore suggest that

TABLE 4. *Substrate and inducer specificity of the energized xylose transport*[a]

Carbon compound in growth medium	Rate of sugar transport (nmol/mg per min)				
	Xylose	Arabinose	Galactose	Fucose	TMG
Glycerol + xylose	4.4	0.3	0.9	0.3	1.9
Glycerol	0.01	0.8	3.1	0.6	2.2
Arabinose	0.3	3.2	1.3	0.6	1.9
Glycerol + ribose	0.2	0.3	0.8	0.4	5.1
Glycerol + fucose	0.3	0.2	2.8	2.4	4.2
Glycerol + glucose	0.1	0.2	0.4	0.1	0.8
Glycerol + xylose	(6.9)	(0.2)	(1.0)	(0.3)	(0.02)
Glycerol + glucose	(0.9)	(0.8)	—[b]	—	(0.3)

[a] *E. coli* strain VL17 was grown on the indicated carbon sources (20 mM glycerol, 10 mM sugar, 1 mM D-fucose) and prepared for transport measurements as described in the text. The figures in parentheses are measurements made with *E. coli* strain K10.

[b] —, Not tested.

the *xyl* lesion of strain VL1 also maps at min 79 on the recalibrated map of Bachmann et al. (3).

The addition of xylose to anaerobic suspensions of strain VL18 grown in the presence of xylose failed to promote an alkaline pH change (Fig. 1, Table 2). This failure was not due to a general dislocation of sugar-H^+ symport, since TMG elicited wild-type rates of effective proton uptake when added to VL18 cells induced for the LacY transport system. The rate of energized xylose uptake into strain VL18 grown in the presence of xylose was low (0.3 nmol/mg per min), similar to the value obtained with uninduced strain VL17.

Thus, the loss of energized xylose transport into strain VL18 is coincident with the loss of xylose-H^+ symport activity, and the lesion maps near *mtl*, the position of the xylose operon. The genes for GalP and AraE sugar-H^+ systems map near 61 min and LacY is near 8 min (3). This further substantiates the presence of a separate xylose-H^+ transport system in *E. coli*, although our experiments do not indicate whether the mutation in strain VL18 is in a regulatory or a structural gene.

Xylose transport into subcellular vesicles of *E. coli*. The study of transport reactions in bacteria has been greatly facilitated by measurements on subcellular vesicles prepared by the method of Kaback (21). Interference by cytoplasmic reactions is eliminated, and the vesicle membranes are accessible to ionophores that cannot penetrate the cell wall of intact bacteria (22).

We have prepared such vesicles from strain VL17 grown in the presence of xylose. No significant transport of xylose occurred into the vesicles unless ascorbate plus phenazine methosulfate was added as respiratory substrate (Table 5). The vesicles lack the cofactors or enzymes or both necessary to make ATP, acetyl phosphate, or phosphoenolpyruvate, but they can generate an electrochemical potential of protons (ΔpH and $\Delta\Psi$) across the membranes when respiring (31, 32). That this proton-motive force (26) is driving xylose transport is supported by the susceptibility of transport to the protonophore uncoupling agents 2,4-dinitrophenol, TCS, and CCCP (Table 5). Ionophore A217, a nigericin-like compound capable of discharging ΔpH but not $\Delta\Psi$ (13), brought about partial inhibition, as did valinomycin, which can discharge the electrical component $\Delta\Psi$, but not ΔpH (13). The two compounds together caused almost complete inhibition (Table 5), presumably by discharging both components of the total proton-motive force.

The occurrence of xylose transport in vesicles, its energization by respiratory substrate, and its inhibition by protonophores and ionophores argue that, like β-galactoside transport on the LacY permease, a sugar-proton symport system is operating.

TABLE 5. *Respiration dependence and uncoupler sensitivity of xylose transport into subcellular vesicles of E. coli*[a]

Addition	Concn	Activity (nmol/mg per min)	Activity (% of control)
None	—	1.0	—
Ascorbate + Phenazine methosulfate	20.0 mM 0.1 mM	13.7	—
Ascorbate + Phenazine methosulfate + Ethanol	20.0 mM 0.1 mM 2% (vol/vol)	16.0	—
2,4-Dinitrophenol	1 mM		27
TCS	10 μM		0
CCCP	10 μM		41
A217‡	4 μg/ml		57
Valinomycin	4 μM		20
A217‡ + Valinomycin	4 μg/ml 4 μM		6

[a] Subcellular vesicles were prepared from glycerol-plus xylose-grown strain VL17 essentially as described by Kaback (21). Radioactive xylose (200 μM) was added to 0.1 or 0.25 mg of vesicle protein in 0.5 ml of 50 mM potassium phosphate, 10 mM $MgCl_2$ (pH 6.6) bubbled with air. Ascorbate and phenazine methosulfate were added immediately before the xylose and were present in all the experiments that included protonophores (added 3 min before the xylose). Samples were filtered 15 s after xylose addition and washed with approximately 4 ml of 100 mM LiCl. Values are the mean of duplicate measurements except those marked with ‡.

DISCUSSION

A xylose transport system in *E. coli* was first characterized by David and Wiesmeyer (8), who reported that it was energy dependent. We have sought to distinguish between three types of mechanism for the xylose transport activity appearing in cells grown on a mixture of glycerol and xylose: energization by a proton-motive force (26–28), direct energization by ATP or acetyl phosphate (4, 5, 18), and phosphorylation by phosphoenolpyruvate (25, 30). The following observations indicate that xylose transport is energized by a proton-motive force.

When xylose was added to anaerobic energy-depleted cells, an uncoupler-sensitive alkaline pH change expected for a sugar-H^+ symport

(chemiosmotic) system occurred. Directly energized systems yield no pH change (7, 16), whereas phosphotransferase systems give an acid shift (P.J.F.H., unpublished observations).

Energized xylose transport was inhibited by uncoupling agents. In our experience both chemiosmotic and directly energized systems are susceptible to uncoupling agents, the latter presumably through a lowering of intracellular ATP and possibly acetyl phosphate (4, 5, 18) although the directly energized systems may be less sensitive (K.R.D., Ph.D. thesis). Phosphotransferase systems are not inhibited by uncoupling agents and may actually be stimulated (17, 24).

Xylose transport was not sensitive to arsenate or fluoride, which is expected if respiration maintains the electrochemical gradient of protons necessary for a chemiosmotic energization. Both directly energized and phosphotransferase mechanisms are susceptible to arsenate (4, 5, 24, P. J. F. Henderson and K. R. Daruwalla, Abstr. 11th FEBS Meet., Copenhagen, abstr. no. B7-2 375 4).

Neither directly energized nor phosphotransferase mechanisms are manifested in subcellular vesicles of *E. coli* prepared by osmotic shock, since the preparative procedure eliminates soluble protein components and the enzymes for generating phosphoenol-pyruvate, ATP, or acetyl phosphate (22). However, xylose transport, dependent on respiration and susceptible to uncoupling agents, occurred in such vesicles. Again, these are characteristics expected of energization by a chemiosmotic mechanism.

It is unlikely that a sugar-Na^+ symport mechanism is involved in xylose transport because the vesicle preparation is free of Na^+ ions. With intact cells the replacement of K^+ by up to 135 mM Na^+ increased the rate of xylose uptake by 50 to 70%. This minor stimulation is probably an unspecific effect since it also occurred for proline (23) and glucose 6-phosphate (10) transport, both of which are reported to operate by a proton symport mechanism.

The substrate and inducer specificity and the genetic linkage with *mtl* show that the xylose transport system described above is distinct from other sugar transport systems of *E. coli*. However, it is possible that this organism can induce a second xylose transport activity, which may be differently energized. The appearance of the xylose transport activity characterized by David and Wiesmeyer (8) was repressed by including glucose or glycerol in the growth medium. Since we routinely grew the organism in the presence of glycerol, it is likely that the xylose transport system described by David and Wiesmeyer (8) would be repressed in our cultures. Furthermore, Shamanna and Sanderson (35) state that xylose uptake into *E. coli* exhibited biphasic kinetics consistent with the presence of two systems, with K_m values of 24 μM and 100 μM. The apparent K_m of the energized xylose transport described above is 23.9 $\pm$ 2.4 μM (K.R.D., Ph.D. thesis). In the closely related enteric bacterium, *Salmonella typhimurium*, xylose transport exhibited a K_m of 410 μM and was probably repressed by glucose (35); we could not detect an alkaline pH change when xylose was added to anaerobic suspensions of xylose-induced *S. typhimurium* (unpublished data). Thus, xylose transport into this organism is quite different from the proton-linked xylose transport occurring in *E. coli*.

To summarize, the xylose transport activity of *E. coli* that is abolished by a mutation mapping near *mtl* is energized by a proton-motive force, as envisaged by Mitchell (27, 28). There may be another separate xylose transport system in *E. coli*, but further studies will be necessary to establish its mechanism of energization.

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