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Evolution of Propanediol Utilization in *Escherichia coli*: Mutant with Improved Substrate-Scavenging Power

ANDREW J. HACKING, JUAN AGUILAR,† AND E. C. C. LIN*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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Wild-type strains of *Escherichia coli* are unable to use L-1,2-propanediol as a carbon and energy source. A series of mutants, able to grow on this compound at progressively faster rates, had been isolated by repeated transfers to a medium containing 20 mM L-1,2-propanediol. These strains synthesize at high constitutive levels a propanediol:nicotinamide adenine dinucleotide oxidoreductase, an enzyme serving as a lactaldehyde reductase during L-fucose fermentation by wild-type cells. In this study, a mutant that can grow rapidly on the novel carbon source was subjected to further selection in a medium containing L-1,2-propanediol never exceeding 0.5 mM to obtain a derivative that has an increased power to extract the substrate from the medium. The emerging mutant exhibited four changes at the enzymatic level: (i) fuculose 1-phosphate aldolase activity is lost; (ii) the constitutive propanediol oxidoreductase activity is increased in its level; (iii) lactaldehyde dehydrogenase becomes constitutive and shows an elevated specific activity in crude extracts; and (iv) at low concentrations of propanediol, the facilitated diffusion across the cell membrane is enhanced. Changes two to four seem to act in concert in the trapping of propanediol by hastening its rate of entry and conversion to an ionized metabolite, lactate.

The acquisition of a catabolic pathway for L-1,2-propanediol by mutant strains of *Escherichia coli* K-12 has been shown to depend on the genetic alteration of the dissimilatory system for L-fucose (4, 14, 15). In wild-type cells, L-fucose is metabolized by the sequential action of fucose permease (14), fucose isomerase (13), fuculose kinase (16), and fuculose 1-phosphate aldolase (10). The products of the last reaction are dihydroxyacetone phosphate and L-lactaldehyde. Under aerobic conditions, L-lactaldehyde is oxidized in two steps to pyruvate by a nicotinamide adenine dinucleotide-dependent lactaldehyde dehydrogenase (34), and a flavin-linked lactate dehydrogenase (4). Under anaerobic conditions, lactaldehyde is reduced to propanediol by a nicotinamide adenine dinucleotide-linked propanediol oxidoreductase (35), and propanediol is lost into the medium (4) (Fig. 1).

Propanediol cannot be utilized by wild-type strains of *E. coli* even if molecular oxygen is available. The failure of induction of the oxidoreductase constitutes at least one obstacle. Mutants isolated after repeated selection on propanediol as the carbon and energy source synthesize the oxidoreductase constitutively and at very high levels of activity. The product formed by this enzyme apparently in turn induces lactaldehyde dehydrogenase, which catalyzes the formation of L-lactate (4). Continued selection on propanediol results inevitably in the noninducibility of the permease, isomerase, and kinase associated with the constitutivity of the aldolase (14, 15). These changes were unforeseen; their possible significance will be dealt with below.

An outstanding question remains: that of cell permeability to propanediol. In the case of a similar compound, glycerol, there is no requirement of a specific transport system for growth on the substrate at concentrations above 5 mM. Below this concentration, facilitated diffusion becomes progressively more important (27). In comparison to glycerol, propanediol has a slightly smaller molecular radius (2.61 nm versus 2.74 nm) (11), a much higher olive oil-water partition coefficient (two orders of magnitude) (5), and four hydrogen-bonding groups instead of six, the number of which correlates negatively to membrane permeability (36). Together these differences should greatly favor propanediol over glycerol as a permeant across phospholipid bilayers. This was indeed found to be true for a number of biological membranes, including the plant cell *Chara ceratophylla* (6), human erythrocyte (11), and frog skin (37), though in retro-

† Present address: Department of Biochemistry, Faculty of Pharmacy, University of Barcelona, Spain.

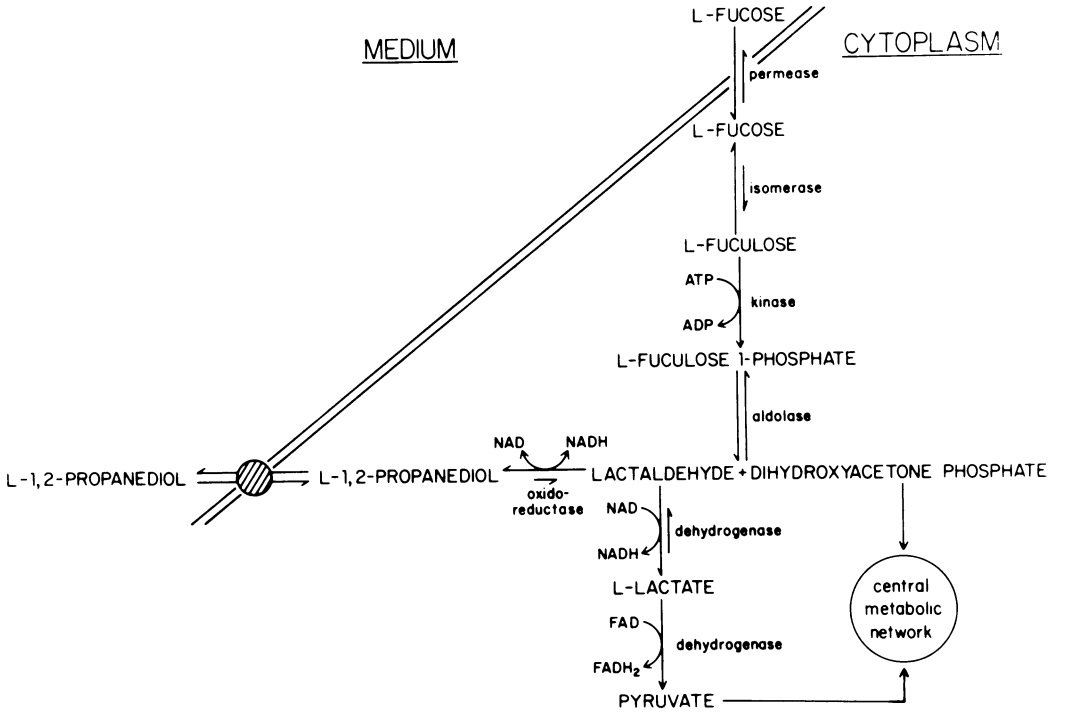

FIG. 1. *Scheme for propanediol and fucose metabolism in E. coli. The enzyme catalyzing the interconversion of propanediol and lactaldehyde is referred to as propanediol oxidoreductase, since the actual role of this protein depends upon the strain in which it is found. FAD, Flavin adenine dinucleotide; and NAD$^+$ and NADH, respectively, the oxidized and reduced form of nicotinamide adenine dinucleotide.*

spect the possible presence of transport proteins in some of the systems might have distorted the measurements to some degree. In any event, it seems quite possible that the rate of nonspecific diffusion of propanediol across the *E. coli* membrane is so much more rapid than that of glycerol as to preclude any necessity for a transport system. One way to approach this problem is to select for mutants that can grow more effectively in media containing limiting concentrations of propanediol and to discover what kind of cellular changes account for the enhanced substrate-scavenging power. For this purpose, cells of strain 3, selected for rapid growth under substrate nonlimiting conditions, were used as the parental population. Throughout this study, 1,2-propanediol (labeled or unlabeled) was added as a racemic mixture. Since it was established in a previous study that only the L-isomer is utilized (35), the concentrations given in this report refer only to that isomer, unless otherwise specified.

MATERIALS AND METHODS

Chemicals. L-Fuculose was obtained from a *Klebsiella aerogenes* mutant that excretes this compound into the medium when supplied with L-fucose (32). L-Fucolose 1-phosphate was prepared enzymatically from L-fuculose (32) and purified (14). L-Lactaldehyde was synthesized by reacting ninhydrin with D-threonine, according to the method of Zagalak et al. (41), and was purified by a modified procedure (14). DL-1,2-Propanediol was purchased from Fisher Scientific Co., Pittsburgh, Pa., and redistilled. DL-1,2-[1-^{14}C]propanediol was purchased from ICN Pharmaceuticals Inc., Irvine, Calif. The labeled compound was incubated with cells of strain 1 to remove nonspecific interfering substances (about 1% of the counts) (40). L-[1-^{14}C]fucose was purchased from Amersham/Searle, Arlington Heights, Ill. L-Fucose was obtained from Pfanstiehl Laboratories, Waukegan, Ill.; casein acid hydrolysate (vitamin-free) was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio; and purified enzymes were obtained from Boehringer Mannheim Biochemicals, New York.

Bacteria and phage. Strain 1 of *E. coli* K-12 served as the wild-type parent (35). Strain 3, able to grow rapidly on 20 mM propanediol, but no longer able to grow on fucose, was isolated from strain 1 after subjecting a population of wild-type cells to ethyl methane sulfonate mutagenesis (21) and culturing the survivors for about 100 mass doublings in a propanediol mineral medium (35). Strain 421, another fucose-negative derivative of strain 1, was selected in a similar manner, but without mutagenesis (15). Strain 430, able to scavenge the novel carbon source effectively, was obtained without mutagenesis by recycling cells of strain 3 in media containing propanediol concentra-

tions of 0.5 mM or less. Each subculture was incubated at 37°C until cessation of growth, and the process was continued for at least 120 mass doublings. Strain 32 (lacking propanediol oxidoreductase) and strain 40 (lacking lactaldehyde dehydrogenase) were derived as propanediol-negative mutants from strain 3 (34, 35). Transduction with phage P1 was carried out by the method of Luria et al. (22). Ampicillin enrichment of ethyl methane sulfonate-induced mutants of strain 430 that lost the ability to grow on propanediol was carried out by the method of Miller (24).

Growth of cells. Carbon sources for aerobic growth were added to a basal inorganic medium (35) in the following concentrations (either alone or in mixtures): L-fucose, 10 mM; propanediol, 20 mM or 0.5 mM; and casein acid hydrolysate, 0.5% (wt/vol). Solid media were prepared by the further addition of agar to 1.5% (wt/vol). Growth at 37°C was monitored with a Klett-Summerson colorimeter with a no. 42 filter, or by estimation of viable count after dilution and plating on enriched agar. The latter method was used when the initial propanediol concentration in the growth medium was 0.5 mM.

Enzyme assays. Cells in late or midexponential phase were collected by centrifugation, washed in 0.1 M sodium phosphate (pH 7), suspended in four times their wet weight of the same buffer containing 2.5 mM glutathione, and sonically disrupted by two 1-min pulses (20 kc/s) in a tube chilled at 0°C. The supernatant fraction, after centrifugation at 100,000 × *g* for 45 min at 0°C (to remove reduced nicotinamide adenine dinucleotide oxidase), was used for enzyme assays. L-Fucose isomerase (EC 5.3.1.3) activity was determined from the initial rate of L-fuculose formation by the cysteine carbazole method (9). The assay of L-fuculose kinase (EC 2.7.1.51) was based on the fuculose-stimulated consumption of ATP (14). The assay of L-fuculose 1-phosphate aldolase (EC 4.1.2.17) was dependent on the formation of dihydroxyacetone phosphate (10). Propanediol oxidoreductase (EC 1.1.1.77) (35) and lactaldehyde dehydrogenase (EC 1.2.1.22) (34) activities were measured spectrophotometrically by the formation of reduced nicotinamide adenine dinucleotide in a final volume of 1 ml. All reaction rates were measured in a Gilford recording spectrophotometer. Protein concentrations were determined by the biuret reagent (12).

Transport assays. L-Fucose permease activity was determined by the initial rate of cellular uptake of L-[1-^{14}C]fucose (14). For the measurement of propanediol uptake, cells harvested in a late exponential phase of growth were washed with mineral medium and suspended at approximately 10^9 cells per ml in the same medium with chloramphenicol added at a concentration of 100 μg/ml. The assay was started by adding 0.5 ml of the suspension to 0.5 ml of mineral medium containing 0.2 μmol of [1-^{14}C]propanediol (0.2 μCi). Samples (0.1 ml) were withdrawn at 1-min intervals, and the uptake reaction was terminated by separating the cells from the incubation medium by filtration through a membrane wetted previously with mineral medium. The filter was immediately washed with 5 ml of this medium and transferred to 5 ml of Bray solution for determination of the radioactivity by scintillation counting. If the filter and cells were allowed to dry before washing, a substantial loss of counts occurred.

Gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed at pH 8.6 as described by Davis (8). Gels at concentrations of 4, 6, and 8% acrylamide (wt/vol) were used. After electrophoresis, the gels were stained for propanediol oxidoreductase activity by incubation in a reaction mixture which contained (per 100 ml) 10 mmol of sodium glycine (brought to pH 9.5 by NaOH), 1 mmol of $MnCl_2$, 2.5 mmol of propanediol, 20 mg of oxidized nicotinamide adenine dinucleotide, 10 mg of 3-(4,5-dimethylthiazol-2)2,5-diphenyl tetrazolium bromide, and 0.2 mg of phenazine methosulfate.

Glycerol dehydrogenase was stained by a similar method, substituting glycerol (1 mmol) for propanediol. Because of cross-specificity of glycerol dehydrogenase and propanediol oxidoreductase, two blue bands appear when either substrate is used. However, the two proteins may be readily identified by the facts that: (i) each enzyme is more strongly stained by its proper substrate, and (ii) propanediol oxidoreductase is present at high constitutive levels in extracts of strain 3, whereas glycerol dehydrogenase is present at a high level in extracts of strain 1 grown anaerobically on glucose (35). After treatment for 30 min in 12% (wt/vol) trichloroacetic acid, the proteins were stained by Coomassie blue, added to a concentration of 0.05%. After overnight incubation at room temperature, the gels were destained in 7% acetic acid and 25% methanol (vol/vol).

Stopped-flow spectrophotometry. Turbidity changes of suspensions of *E. coli* cells after exposure to hypertonic solutions were measured in a stopped-flow spectrophotometer as described by Alemohammad and Knowles (1). Cells suspended in 10 mM imidazole buffer (pH 7) to give an absorbance of 2.0 (680 nm in a 1-cm cuvette) were mixed with an equal volume of NaCl, glycerol, or DL-1,2-propanediol in the same buffer, and the change in transmittance was displayed on a storage oscilloscope. The light traces were photographed, and measurements were made from the prints.

RESULTS

Growth properties of strain 430. On agar containing 0.5 mM propanediol, colonies of strain 430 grew faster than those of the parent strain 3 or of strain 421, which was independently isolated from wild-type strain 1 after repeated transfer into a medium with 20 mM substrate (15). A threefold difference in diameter could be observed. In liquid medium, strain 430 showed a lower growth K_m (1 mM) than strain 3 (7 mM). In liquid medium containing 20 mM propanediol, however, the three strains showed similar mass doubling times in the range of 130 to 140 min.

Like the parent, strain 430 was unable to utilize L-fucose either aerobically or anaerobically but retained wild-type growth patterns on the isomer L-rhamnose. The characteristics of strain 430 remained stable after months of re-

peated subculture on nonselective media. No spontaneous fucose-positive revertants of strain 430 were detected among the 10^{10} cells screened, whereas a reversion frequency of 5×10^{-7} was obtained with strain 3 (14).

Levels of the fucose enzymes. In wild-type strain 1, fucose permease, fucose isomerase, fuculose kinase, and fuculose 1-phosphate aldolase were all inducible. In strain 3, the first three proteins were noninducible, but fuculose 1-phosphate aldolase, even though thereby becoming gratuitous, was synthesized constitutively (14). In strain 430, all of the four activities were absent (Table 1). Moreover, lactaldehyde dehydrogenase became constitutive, and the constitutive propanediol oxidoreductase level became almost twice that found in strain 3.

Properties of propanediol oxidoreductase in strains 1, 3, and 430. A preliminary characterization of this enzyme in cell extracts failed to reveal any evidence for structural mutations in strain 430 or in its predecessor, strain 3. In an experiment involving heat inactivation, the enzyme from cells of the wild-type strain 1 lost 50 to 60% of the activity after incubation for 10 min at 50°C. The inactivation rates at 45, 48, and 50°C were very similar for the enzymes prepared from strains 1, 3, and 430. The active enzymes from the three strains showed an identical rate of migration during polyacrylamide gel (4, 6, or 8%) electrophoresis in a nondissociating buffer system at pH 8.6. Also, no striking difference in K_m to propanediol was detected (Table 2). Although these determinations were carried out with crude extracts, the results are in close agreement to the measurements made with a 20-fold purified enzyme preparation from strain 3 (35).

Propanediol uptake and enzymatic composition. When cells of strain 1 were grown aerobically on fucose, their lactaldehyde dehydrogenase, but not propanediol oxidoreductase, became highly induced (14). Such cells retained very little radioactivity when incubated with 0.2 mM labeled propanediol (Table 3). On the other hand, when cells of strain 1 were grown anaerobically on fucose, their propanediol oxidoreductase, but not lactaldehyde dehydrogenase, became highly induced (14). Such cells retained no radioactivity when incubated with labeled propanediol. When cells of strain 3 were grown aerobically on either casein hydrolysate or propanediol, both their lactaldehyde dehydrogenase and propanediol oxidoreductase were produced at high levels. Such cells showed active uptake of the substrate. In mutants derived from strain 3, but lacking either propanediol oxidoreductase (strain 32) or lactaldehyde dehydrogenase (strain 40), no propanediol uptake by cells grown on casein hydrolysate was observed, in confirmation of a previous observation (T. T. Wu, personal communication). Presumably because neither propanediol nor lactaldehyde can be retained effectively against a concentration gradient by the cell, the only way to prevent the

TABLE 2. *Kinetic properties of propanediol oxidoreductase in strains 1, 3, and 430*[a]

Strain	Growth conditions	K_m for L-1,2-propanediol (mM)	V_{max} (nmol × min^{-1} × mg of protein^{-1})
1	Fucose (anaerobic)	0.6	300
3	CAA[b] (aerobic)	0.9–1.1	740
430	CAA (aerobic)	0.6	1,700

[a] Assays were carried out with crude cell extracts.
[b] Casein acid hydrolysate.

TABLE 1. *Activities of fucose pathway enzymes in extracts of strain 430 and ancestors grown on different media*

Strain	Carbon source	Fucose permease[a]	Fucose isomerase[b]	Fuculose kinase	Fuculose 1-phosphate aldolase	Propanediol oxidoreductase	Lactaldehyde dehydrogenase
1[c]	CAA[d]	0	36	27	0	20	93
	CAA + fucose	55	500	440	240	80	100
	Fucose	58	520	420	130	42	280
3[c]	CAA	0	43	80	490	410	130
	CAA + fucose	0.08	30	46	490	390	110
	Propanediol		43	57	490	420	260
430	CAA				0	750	380
	CAA + fucose	0	20	48	0	680	420
	Propanediol				0	720	410

[a] Expressed in nanomoles × minute^{-1} × milligram of dry weight^{-1} at 37°C.
[b] Specific activities of all the enzymes are expressed in nanomoles × minute^{-1} × milligram of protein^{-1}.
[c] Data from reference 14.
[d] Casein acid hydrolysate.

TABLE 3. *Uptake of [1-^{14}C]propanediol by various strains*

Strain	Carbon source for growth	0.2 mM propanediol uptake (nmol × min^{-1} × mg of dry wt^{-1})
1	CAA	0
	Fucose (aerobic)	0.5
	Fucose (anaerobic)	0
3	CAA	3.5
	Propanediol	3.1
32	CAA	0
40[a]	CAA	0
421[b]	CAA	3.2
	Propanediol	4.1
430	CAA	7.3
	Propanediol	7.5

[a] See reference 34 for full description of the strain.
[b] See reference 15 for full description of the strain.

loss of substrate is to convert it to a charged metabolite, lactate.

Cells of strain 430, irrespective of whether they were grown on casein hydrolysate or propanediol, exhibited uptake rates approximately double those of strain 3. This enhanced substrate uptake power of strain 430 cannot be accounted for solely by the increment in the activity of propanediol oxidoreductase or by that of lactaldehyde dehydrogenase. First, strain 421 (selected on 20 mM propanediol [15]), with a propanediol oxidoreductase activity three times higher than that of strain 3, was not superior to strain 3 in propanediol uptake. Secondly, a twofold induction of lactaldehyde dehydrogenase in strain 3 itself by growth on propanediol (14) did not elicit a significant increase in the rate of substrate uptake (Table 3).

The synergistic action of the two enzymes catalyzing reactions in tandem is probably critical. In addition, as will be shown in a further section, an important change also occurred in strain 430 at the level of the cell membrane.

Stopped-flow spectrophotometry. The permeability of cell membranes to propanediol was first explored by a technique based on the plasmolysis of cells when exposed to a hypertonic solution. This method was applied with success to measure the catalyzed permeation of glycerol across the *E. coli* membrane. The inducible or constitutive synthesis of the glycerol facilitator protein increased the rate of glycerol penetration into the cells. With propanediol as the permeant, however, the half-time of equilibration across the cell membrane of strains 1, 3, and 430 was found to be approximately 0.1 s or less, which is too rapid to be measured with the apparatus. This is true whether the cells were grown on glucose, casein hydrolysate, fucose (strain 1), or propanediol (strains 3 and 430), but the findings cannot be extrapolated to concentrations in the millimolar range. Another means must therefore be found to test for the existence of a transport system acting on propanediol.

Inhibition of propanediol uptake by sugars. The specific transport of a number of carbohydrates can be inhibited by sugars such as glucose, which is translocated across the cell membrane by vectorial phosphorylation with phosphoenolpyruvate as phosphoryl group donor (the phosphotransferase system or PTS). This inhibition is believed to be mediated by an interaction between the target permease and the sugar-specific component of the PTS in complex with its substrate. The effector molecule needs not to be metabolizable. For example, α-methylglucoside, which can be phosphorylated but not utilized, acts like glucose (2, 18, 19, 23, 29, 30, 31, 38).

Since conventional uptake assays failed to reveal an active concentration of labeled propanediol in strain 3 derivatives that had lost propanediol oxidoreductase activity, and the osmotic assay failed to reveal facilitated diffusion in strains 1, 3, and 430, the inhibitability of propanediol uptake by a PTS substrate was tried as a third resort to test for mediated transport of propanediol. Figure 2A shows that 0.5 mM α-methylglucoside indeed severely inhibited (90%) the uptake of labeled propanediol by cells of strain 430, if they were first supplied with glucose for 1 h to induce the synthesis of the specific membrane protein (a longer period of induction was avoided to minimize catabolite repression of the propanediol system during growth). Milder inhibition (60%) was observed when the cells were not preinduced. These results are consistent with the partial constitutivity of the PTS for glucose in this strain of *E. coli* (unpublished observation). With 0.5 mM mannitol as a test compound, inhibition was 90% after preinduction but less than 20% without such treatment (Fig. 2B). The results in this case are consistent with the high induction ratio of the PTS for mannitol (33). Similar patterns of inhibition were obtained with propanediol uptake by strain 3 (data not presented).

When tested with cell extracts, activities of propanediol oxidoreductase and lactaldehyde dehydrogenase were not affected by α-methylglucoside (5 mM) either at pH 7.5 or at the higher pH's of the standard assays. This is true regardless of whether or not the cells were preinduced with glucose.

The presence of 5 mM α-methylglucoside in cultures of strains 3 or 430 growing on 20 mM propanediol (a concentration that should render

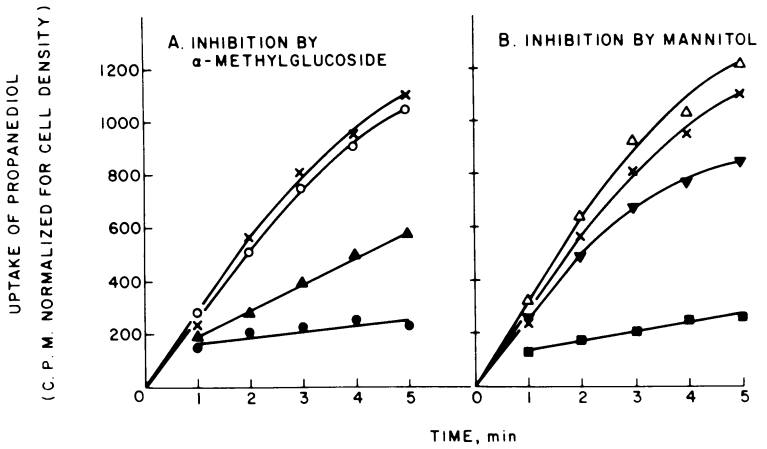

FIG. 2. *Inhibition of [1-^{14}C]propanediol uptake by α-methylglucoside and mannitol. Four cultures of strain 430 were grown in a medium containing 20 mM propanediol. One hour before harvesting, glucose (5 mM) was added to one culture and mannitol (5 mM) to another. The collected cells were resuspended and assayed for the rate of [1-^{14}C]propanediol uptake at 0.2 mM. (A) Control cells* (×) *and glucose-induced cells* (○) *incubated with labeled substrate alone. Control cells* (▲) *and glucose-induced cells* (●) *incubated with substrate plus 0.5 mM α-methylglucoside. (B) Control cells* (×) *and mannitol-induced cells* (△) *incubated with substrate alone. Control cells* (▼) *and mannitol-induced cells* (■) *incubated with substrate plus 0.5 mM mannitol.*

a specific membrane transport unnecessary) did not inhibit growth. In a parallel experiment, growth of strain 1 on fucose, however, was inhibited.

It thus seems likely that membrane carriers intervene for the facilitated diffusion of propanediol across the cell membrane when the substrate is presented at low concentrations.

Selection of mutants impaired in propanediol uptake. Since the colony size of strains 3 and 430 was clearly distinguishable on agar with propanediol at 0.5 mM, but not at 20 mM, an attempt was made to identify mutants of strain 430 defective in propanediol permeation on the basis of this criterion. After mutagenesis of a population of strain 430, the cells were grown in liquid medium supplied with 20 mM propanediol. The population was then subjected to two cycles of ampicillin selection in a nonpermissive medium with 0.5 mM propanediol as the sole source of carbon and energy. Survivors were spread on rich medium and replica printed on plates with 0.5 and 20 mM propanediol. Isolates with apparently poor growth only on agar with 0.5 mM propanediol were recloned for verification of the presumptive phenotype. Of 250 colonies thus screened, 10 independent mutants of the desired characteristics were obtained. Three subphenotypes, represented by strains 431, 434, and 440, were found. Strain 431 suffered losses in both the uptake and the oxidoreductase activities. Strain 434 was unaltered in the ability to take up propanediol but was reduced in propanediol oxidoreductase activity. Strain 440 was reduced in the ability to take up propanediol but was unaltered in the oxidoreductase activity. All three strains retained the high constitutive levels of lactaldehyde dehydrogenase (Table 4; refer also to Tables 1 and 3) and remained noninducible in the fucose pathway (data not presented).

The propanediol uptake by all these mutants was still inhibitable by α-methylglucoside, though the degree of inhibition in strain 440 was less evident because of its low initial activity.

The properties of strain 430 and its derivatives strengthen the notion that a specific membrane transport mechanism contributes to the uptake process of propanediol. Moreover, a critical propanediol oxidoreductase activity of about 500 U seems to be required for effective trapping of the carbon source under growing conditions.

Transduction. A preliminary analysis of strain 430 was undertaken to discover if the enhanced permeability to propanediol, the increased level of propanediol oxidoreductase, the constitutivity of lactaldehyde dehydrogenase, and the absence of fuculose 1-phosphate aldolase are traits that can be readily separated. Cells of wild-type strain 1 were transduced, with phage P1 grown on strain 430, for the ability to grow on agar supplied with 20 mM propanediol. When 200 of such transductants were tested for growth on fucose, all were negative (Table 5). All grew as small colonies on agar containing 0.5 mM propanediol. Enzymatic analysis of two trans-

ductants revealed a profile indistinguishable from that of strain 430. Their rates of propanediol uptake, however, were close to that of strain 3. The low uptake activity remained sensitive to inhibition by α-methylglucoside.

If the earlier transductional analysis of strains 3 and 421 (15) is also considered, it may be concluded that all the mutations affecting the fucose pathway and the ability to utilize propanediol are closely linked, with the exception of that conferring the improved propanediol uptake in strain 430. It is very likely that another permeation system belonging to a different metabolic pathway was mobilized by the strain.

The unknown origin of the second propanediol transport component. An attempt was made to reveal the normal role of the second permeation component by exploring the ability of several carbohydrates to act as inhibitors of propanediol uptake by strain 430. A number of pentoses and hexoses that can serve *E. coli* K-12 as carbon and energy source (26), but whose capture does not involve phosphorylation by the PTS, were tested at 0.5 mM. No significant effect was observed with L-arabinose, L-fucose, D-galactose, glycerol, L-rhamnose, D-ribose, and D-xylose. Moreover, strain 430 retained the ability to grow on all of these compounds save L-fucose. The possible recruitment of glycerol facilitator by its constitutive synthesis was made unlikely also by the observation that glycerol kinase, whose structural gene belongs to the same operon as the gene for the facilitator protein, remained inducible.

TABLE 4. *Selection from strain 430 of three classes of mutants giving small colonies on 1 mM propanediol*

Mutant	Carbon source for growth	Propanediol[a] uptake	Propanediol[b] oxidoreductase	Lactaldehyde dehydrogenase
430	CAA		750	380
	Propanediol	7.5	720	410
431	CAA		260	440
	Propanediol	4.4	220	450
434	CAA		220	390
	Propanediol	8.0	250	450
440	CAA		660	500
	Propanediol	3.2	750	480

[a] Expressed in nanomoles × minute^{-1} × milligram of dry weight^{-1}.

[b] Enzyme activities in nanomoles × minute^{-1} × milligram of protein^{-1}.

TABLE 5. *Transduction of fucose and propanediol markers*

Donor	Recipient	Phenotype selected	Cotransduction of unselected marker
Strain 430 (*fuc prd*$^+$)	Strain 1 (*fuc*$^+$ *prd*)	Prd$^+$	Fuc$^-$ 200/200

DISCUSSION

In studies on acquisitive evolution with bacteria as model systems, a number of genetic changes that confer novel or enhanced metabolic abilities have been encountered. These include the constitutive synthesis of an enzyme with a side specificity for the novel substrate, a shift in the catalytic property of the constitutive enzyme, an increased production of the constitutive enzyme, and the mobilization of a permease by derepression (3, 17, 20, 28). Variations of the conditions of mutagenesis or selection can favor one strategy over another. Thus, in experiments on the acquisition of a pathway for xylitol utilization by *K. aerogenes*, mutants producing dehydrogenases with improved apparent affinities for the novel substrate emerged after nitrosoguanidine mutagenesis, but mutants producing elevated levels of the original enzyme emerged under the influence of UV irradiation (28, 40). Selection at low concentrations of xylitol favored a mutant that produced constitutively a permease for D-arabitol with fortuitous activity on xylitol (40). In the present work, the outgrowth of strain 430 under conditions that require high scavenging power of the cell provides another example of the mobilization of a membrane carrier for a new function.

From the sequence of phenotypic changes that occurred during the course of selection, it seems that the evolution of the propanediol pathway was operating under two special constraints. One is apparently related to the nature of the regulatory mechanisms controlling the expression of the genes for fucose dissimilation, whereas the other is probably related to the relatively high nonspecific permeability of cellular membranes to the substrate and its immediate product.

Under the first constraint, the noninducibility of fucose permease, fucose isomerase, and fuculose kinase might be an unavoidable mechanistic consequence of certain mutations that permit an increased expression of the propanediol oxidoreductase gene under aerobic conditions (14, 15). The loss of fuculose 1-phosphate aldolase might have occurred for the same reason, although two other possibilities can be offered: (i) the synthesis of this protein being gratuitous, the elimination of the wasteful process by a mutation conferred an advantage under conditions of semistarvation, and (ii) the activity of this enzyme caused deleterious accumulation of fuculose 1-

phosphate, an ill effect which was prevented by a mutation.

Under the second constraint of minimizing metabolic loss, both the increase in the activity of propanediol oxidoreductase and that of lactaldehyde dehydrogenase were important for the rapid conversion of an uncharged small molecule into a retainable ionic compound, lactate (see reference 7 for the development of this concept). The constitutivity of lactaldehyde dehydrogenase is likely to be the most readily available way for raising the cellular activity of this enzyme, as was the case with the oxidoreductase. Although effective metabolite retention is a necessary condition for cellular economy, this is not sufficient to assure an adequate rate of supply. When the external substrate concentration is low, an adaptive feature of equal import is the recruitment of a membrane carrier, even for a nonspecifically permeable compound. The existence of such a membrane transport escaped notice in prior studies of strain 3 and similar mutants and would not have attracted attention had it not been that strain 430 was especially selected for substrate-extracting power. Evidently, the new mutation harnessed a second permeation system, thus augmenting the entry rate of propanediol.

The co-transducibility of the gene for one of the systems that facilitates the permeation of propanediol with the gene of the fucose pathway points to a role of the membrane protein in the pathway itself. In wild-type cells, the protein might serve to catalyze the exit of propanediol during fucose fermentation, and in this respect it may be worthwhile mentioning that fuculose kinase is inhibitable by propanediol (T. T. Wu, unpublished data). Without catalyzed exit, propanediol might accumulate to a harmful level during anaerobiosis.

Facilitated diffusion has been reported in a wide range of cell types and for a variety of metabolites, including sugars, amino acids, and inorganic ions. Little attention has been paid to systems that facilitate the diffusion of propanediol or other small nonpolar molecules, with the exception of a system in human erythrocytes which was found to be active on glycerol and to lesser extents on ethylene glycol and propanediol (36). This neglect is probably due to the one-time prevailing impression that the high permeability of biological membranes to such molecules makes special carriers superfluous. But it should be remembered that the rate of net diffusion in any one direction is dependent upon both the absolute concentration in the source compartment and the gradient across the boundary. Strain 430 was selected under conditions that provided both a low external concentration of propanediol and a low concentration gradient across the cell membrane. It is therefore not unreasonable that the rate of propanediol entry could be significantly accelerated by membrane carriers; and it would not be surprising that special carriers exist for a number of other small and nonpolar molecules in various kinds of cells. The report that ethanol is concentrated by cells of *Corynebacterium acetophilum* by a transport system showing half saturation below 0.1 mM is a good example (25).

Finally, the constitutive presence of even one of the permeation systems for propanediol opens up new evolutionary potentials towards the acquisition of other pathways. Thus, strain 3 was observed to give rise to mutants that grow on D-arabitol, which enters via the "propanediol permease," whereas no success was met with the wild-type strain, even with heavy mutagenesis, because the permease was not inducible by the five-carbon compound. Presumably the probability of having both a constitutive mutation that derepresses the permease and another mutation that permits the internal metabolism of D-arabitol is practically nil. The D-arabitol-positive mutants, interestingly, concentrate the novel carbon source against a gradient (39). Further examination of the substrate specificity of the membrane transport system of such mutants might help to establish the original role of the membrane protein in strain 3.

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