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Experimental Evolution of a Metabolic Pathway for Ethylene Glycol Utilization by *Escherichia coli*

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Spontaneous mutants of *Escherichia coli* able to grow on ethylene glycol as a sole source of carbon and energy were obtained from mutants that could grow on propylene glycol. Attempts to obtain ethylene glycol-utilizing mutants from wild-type *E. coli* were unsuccessful. The two major characteristics of the ethylene glycol-utilizing mutants were (i) increased activities of propanediol oxidoreductase, an enzyme present in the parental strain (a propylene glycol-positive strain), which also converted ethylene glycol into glycolaldehyde; and (ii) constitutive synthesis of high activities of glycolaldehyde dehydrogenase, which converted glycolaldehyde to glycolate. Glycolate was metabolized via the glycolate pathway, which was present in the wild-type cells; this was indicated by the induction in ethylene glycol-grown cells of glycolate oxidase, the first enzyme in the pathway. Glycolaldehyde dehydrogenase was partially characterized as an enzyme of this new metabolic pathway in *E. coli*, and glycolate was identified as the product of the reaction. This enzyme used NAD and NADP as coenzymes, although the NADP-dependent activity was about 10 times lower than the NAD-dependent activity. Uptake of [^{14}C]ethylene glycol was dependent on the presence of the enzymes capable of metabolism of ethylene glycol. Glycolaldehyde and glycolate were identified as intermediate metabolites in the pathway.

One method of determining the molecular mechanisms involved in the evolution of metabolic pathways in microorganisms is experimental enzyme evolution. A microbial population is subjected to a strong selective pressure, and the emerging mutants are characterized genetically and biochemically (5, 23). A well-documented model of experimental evolution is provided by the new metabolic system responsible for the progressive utilization of L-1,2-propanediol (propylene glycol) by mutants of *Escherichia coli* (3, 12, 20). In these mutants, propanediol oxidoreductase is synthesized constitutively and transforms propylene glycol to lactaldehyde, which is subsequently metabolized into lactate and pyruvate.

Wild-type strains of *E. coli* are not able to use ethylene glycol; however, several bacterial species can use this compound as a sole source of carbon and energy (4, 10, 14, 21; J. G. Morris, Biochem. J. **90**:25 p, 1964). The evolution of a new metabolic pathway for ethylene glycol utilization in *E. coli* was considered since (i) *E. coli* K-12 does not grow on ethylene glycol and it is not possible, even after heavy mutagnesis, to isolate mutants that are able to utilize this compound; (ii) propylene glycol-utilizing mutants (see above) produce high constitutive levels of propanediol oxidoreductase, which converts ethylene glycol into glycolaldehyde (2, 3); and (iii) *E. coli* K-12 is able to use glycolate as a sole source of carbon and energy (13, 15, 18).

Thus, a mutation in the propylene glycol-utilizing mutants resulting in the production of an enzymatic activity that is able to convert glycolaldehyde to glycolate would also permit the utilization of ethylene glycol (Fig. 1).

In this report we describe the isolation of such mutants and the characterization of the newly evolved catabolic pathway.

MATERIALS AND METHODS

Bacteria and phage. The wild-type strain used was *E. coli* K-12 strain E-15 (1); we referred to this strain as strain 1. Strain 3 was derived from strain 1 as a propanediol oxidoreductase mutant after ethyl methane sulfonate mutagenesis (20). These two strains were kindly provided by E. C. C. Lin, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Mass. Strains 1 and 3 had unexpectedly lost the ability to grow on glycolate. Strains G1 and G3 were spontaneous mutants of strains 1 and 3, respectively, which had regained the ability to grow on glycolate, although neither of them was able to grow on ethylene glycol. Strain EG3 was derived from strain G3 by its ability to grow on ethylene glycol. Transduction with phage P1 was carried out by the method of Miller (17).

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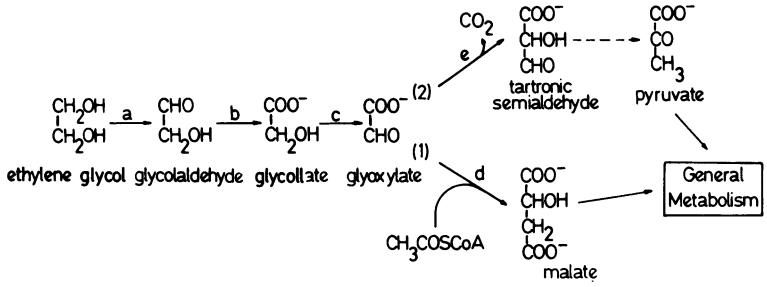

FIG. 1. Reactions involved in ethylene glycol metabolism in *E. coli*. Glyoxylate is the key intermediate between the oxidative and anaplerotic pathways. The following enzymes are involved: propanediol oxidoreductase (a), glycolaldehyde dehydrogenase (b), glycolate oxidase (c), malate synthase (d), and glyoxylate carboligase (e).

Chemicals. DL-1,2-Propanediol was obtained from Merck, Darmstadt, West Germany, and was purified by distillation; 2,6-dichlorophenolindophenol was also obtained from Merck. Ethylene glycol and glycolate were purchased from Carlo Erba Milan, Italy. Glycolaldehyde, glyoxylate, Nitro Blue Tetrazolium, phenazine methosulfate, NAD, and NADP were obtained from Sigma Chemical Co., St. Louis, Mo. [1,2-^{14}C]ethylene glycol was obtained from New England Nuclear Corp., Boston, Mass., and [1,2-^{14}C]glycolate was obtained from the Radiochemical Center, Amersham, England.

Media and culture conditions. The components of the basal inorganic medium have been described previously (2). Unless otherwise stated, the concentrations of the carbon sources in the basal medium were 30 mM ethylene glycol, 30 mM glycolate, and 0.5% (wt/vol) casein acid hydrolysate. For solid media, agar was added at a concentration of 1.5% (wt/vol). Growth was at 37°C in 1-liter Erlenmeyer flasks containing 200 ml of medium and swirled at 250 rpm on a rotary shaker. Growth was monitored with a Klett-Summerson colorimeter equipped with a no. 42 filter.

Isolation of ethylene glycol-positive mutants. Each of nine clones of parent strain G3 was inoculated into one of nine 1-liter Erlenmeyer flasks containing 200 ml of mineral medium supplemented with 30 mM ethylene glycol and 1 mM glucose. Two additional flasks without ethylene glycol were inoculated as controls. All flasks were incubated aerobically at 37°C, and after 24 h a growth of approximately 30 Klett units, due to glucose consumption, appeared in all flasks. After 8 to 10 days, further growth (200 Klett units) was apparent in the nine flasks containing ethylene glycol but not in the control flasks, indicating that the second phase of growth was a consequence of glycol utilization. Clones from each flask were isolated on solid medium containing ethylene glycol as the sole source of carbon and energy, streaked three more times on this solid medium, and finally stored on slants. One of these clones, strain EG3, was selected because of its better growth on solid medium as well as in liquid medium.

Preparation of cell extracts and enzyme assays. Cell extracts were prepared as described previously (2), except that 10 mM phosphate buffer (pH 7.0) was used.

Spectrophotometric assays for propanediol oxidoreductase acting on ethylene glycol were performed at 25°C after NADH formation at 340 nm. Activity was measured in an assay mixture (1 ml) that consisted of 250 mM ethylene glycol, 0.33 mM NAD, and 100 mM sodium glycine buffer (pH 9.5).

NAD- and NADP-dependent glycolaldehyde dehydrogenase activities were measured by following the increase in absorbance at 340 nm. The NAD-dependent activity was measured in an assay mixture that consisted of 1 mM glycolaldehyde, 2.5 mM NAD, and 100 mM sodium glycine buffer (pH 9.5). The NADP-dependent activity was measured in an assay mixture that consisted of 1.5 mM glycolaldehyde, 100 mM KCl, 1 mM NADP, and 100 mM sodium glycine buffer (pH 9.5).

Glycolate oxidase activity was determined in a 30% $(NH_4)_2SO_4$ precipitate fraction of cell extract by measuring the decrease in absorbance at 620 nm caused by the reduction of 2,6-dichlorophenolindophenol coupled to glycolate oxidation (8, 24). The assay mixture consisted of 10 mM glycolate, 10 mM KCN, 0.0012% 2,6-dichlorophenol-indophenol, and 100 mM phosphate buffer (pH 7.0).

Substrate was omitted from the blank mixture in each assay. All reactions were started by adding the enzyme. The concentration of protein was determined by the biuret method (11), using bovine serum albumin as the standard. Units of enzyme activity are expressed as micromoles per minute at 25°C.

Chromatographic techniques. Ethylene glycol, glycolaldehyde, and glycolate were separated by ascending chromatography, using Whatman no. 1 (Chroma) filter paper. Samples were spotted onto paper (17.5 by 18.0 cm) that was chromatographically prewashed in solvent and developed in a saturated chromatography chamber, using *n*-propanol–ammonia (8:2, vol/vol) as the solvent. After the filter paper was air dried, ethylene glycol, glycolaldehyde, and glycolate were stained by spraying with 0.05 N $AgNO_3$ in 2.5 N NH_4OH; the color was developed by heating at 105°C for a few minutes. Glycolate was sometimes identified by a more specific method; the paper was dipped in a solution of acridine (0.1%, wt/vol) in 99.5% ethanol and immediately blotted. After the paper was dried in air, glycolate appeared as a yellow spot that fluoresced under UV light.

For ion-exchange column chromatography, 2.5 ml of cellular extract that contained 50 mg of protein was applied to a column (2.5 by 10 cm) of DEAE-Sephadex

previously equilibrated with 10 mM Tris-hydrochloride–175 mM NaCl (pH 7.3). After the excluded protein was washed off, the retained protein was eluted by using 175 to 500 mM NaCl gradient in 400 ml of the same buffer; 340 mM NaCl eluted glycolaldehyde dehydrogenase under these conditions.

Gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed at pH 7.5 by using 7.5% (wt/vol) acrylamide gels as described by Gabriel (9). After electrophoresis, the gels were stained for NAD-linked glycolaldehyde dehydrogenase activity by incubation at 37°C in a reaction mixture which contained (per liter) 125 mmol of glycine (brought to pH 9.5 by NaOH), 1.3 mmol of glycolaldehyde, 3.3 mmol of NAD, 300 mg of Nitro Blue Tetrazolium, and 100 mg of phenazine methosulfate. Staining for NADP-linked glycolaldehyde dehydrogenase was performed by incubation in a reaction mixture that contained (per liter) 125 mmol of glycine (brought to pH 9.5 by NaOH), 2.0 mmol of glycolaldehyde, 0.6 mmol of NADP, 130 mmol of KCl, 300 mg of Nitro Blue Tetrazolium, and 100 mg of phenazine methosulfate.

Uptake of [^{14}C]ethylene glycol. Cells harvested at the late exponential phase of growth were washed in mineral medium and suspended at a density of approximately 10^9 cells per ml in the same medium. The assay was started by adding 0.75 ml of prewarmed cell suspension to 0.75 ml of mineral medium containing 2.0 μmol of [^{14}C]ethylene glycol (2 μCi/μmol) per ml. Samples (0.2 ml) were withdraw at different times, filtered through a membrane filter (0.45 μm; Millipore Corp., Bedford, Mass.) previously wetted with mineral medium, and immediately washed with 10 ml of this medium at 4°C. The filter was immediately transferred to 5 ml of Bray solution for determination of radioactivity by scintillation counting.

Identification of radioactive intermediates. [^{14}C]ethylene glycol was taken up into the cells as described above; 0.55 ml of cell suspension was incubated with 0.55 ml of mineral medium containing 2.5 μmol of [^{14}C]ethylene glycol (4.0 μCi/μmol) per ml. After 2 min, glycolate or glyoxylate was added to the incubation mixture to a concentration of 25 mM, and incubation was continued for 90 min. The mixture (1 ml) was centrifuged, the cells were suspended in 1 ml of mineral medium, and ethanolic extracts were prepared as described by Dunstan et al. (7). The residue obtained by this procedure was dissolved in 0.025 ml of aqueous 20% (vol/vol) ethanol and spotted onto Whatman no. 1 (Chroma) filter paper.

The radioactive compounds were separated by ascending chromatography as described above. After the chromatograms had been dried, the strips were cut into 3-mm pieces and transferred to 5 ml of 0.5% 2,5-diphenyloxazole in toluene for determination of radioactivity. A mixture of standard ethylene glycol (100 mg/ml) and glycolaldehyde (50 mg/ml) made in aqueous 20% (vol/vol) ethanol was run in parallel and stained with $AgNO_3$, which indicated the relative positions of these compounds. [^{14}C]glycolate was also used as a standard in the marker strip to determine the position of this compound.

RESULTS

Ethylene glycol-positive mutants. Spontaneous mutants able to grow on ethylene glycol as a sole source of carbon and energy were isolated from a propylene glycol-positive strain (strain G3), whereas it was apparently impossible, even after heavy mutagenesis with ethyl methane sulfonate, to obtain such mutants from wild-type strain G1, which is propylene glycol negative (Aguilar, unpublished data).

Of nine independent mutants, one, strain EG3, was selected as the strain which grew best on solid medium containing ethylene glycol, as well as in liquid medium (doubling time, 390 min in medium containing 50 mM ethylene glycol). By plotting the doubling time versus the reciprocal of ethylene glycol concentration, a growth K_m of 35 mM for this compound was calculated. The growth yields of strain EG3 with different concentrations of ethylene glycol were similar (i.e., within 10%) to the growth yields obtained with equivalent carbon concentrations of glucose. The yield was about 10^9 bacterial cells per ml in a medium containing 0.2% carbon source.

Propanediol oxidoreductase in ethylene glycol-positive mutants. As expected, propanediol oxidoreductase activity when ethylene glycol was used as the substrate was very low in the wild-type strain and was present at high levels in strain G3; strain EG3 displayed even higher propanediol oxidoreductase activity (Table 1). Under all conditions, the activities on medium containing ethylene glycol matched the activities obtained when propylene glycol was used as the substrate.

The possibility that ethylene glycol oxidation was a function of an enzyme other than propanediol oxidoreductase was studied by transductional analysis. It is known that propylene glycol-utilizing mutants (strain 3 and, consequently, its derivatives, strains G3 and EG3) lose the ability to grow on fucose and that recovery of fucose utilization by transduction is linked to the loss of propanediol utilization through the inability to produce propanediol oxidoreductase constitutively (6). The separation of propylene glycol and ethylene glycol utilization traits was analyzed by transducing cells of strain EG3 with phage P1 grown on strain G1 and selecting for fucose-positive trans-

TABLE 1. Activities of the ethylene glycol pathway enzymes in several strains grown on casein acid hydrolysate

Strain	Enzyme activities (U/mg of protein)	
	Propanediol oxidoreductase[a]	Glycolaldehyde dehydrogenase[b]
G1	0.005	0.145
G3	0.130	0.165
EG3	0.415	0.560

[a] Ethylene glycol was used as the substrate.
[b] NAD-linked activity.

ductants. When 120 of such transductants were tested for growth on propylene glycol, 98 had lost the ability to grow on this carbon source and simultaneously had lost the ability to grow on ethylene glycol. The 22 that retained the ability to grow on propylene glycol also retained the ability to grow on ethylene glycol.

Glycolaldehyde dehydrogenase in ethylene glycol-positive mutants. Glycolaldehyde dehydrogenase activity in wild-type cells was appreciable but was fourfold higher in the ethylene glycol-positive mutant, strain EG3 (Table 1).

Two glycolaldehyde dehydrogenating activities (an NAD-dependent activity and an NADP-dependent activity) were found to be present in strain EG3, although the NADP-dependent activity was about 10 times lower than the NAD-dependent activity. Experimental evidence suggested that one enzyme was responsible for both activities. Ion-exchange chromatography of an extract of strain EG3 showed that the NAD- and NADP-dependent activities eluted together from the column as a single peak which showed the same ratio of NAD activity to NADP activity as found in the crude extract. In addition, polyacrylamide gel electrophoresis of extracts developed under non-dissociating conditions and further stained for NAD- or NADP-dependent glycolaldehyde dehydrogenase activity resulted in a well-defined band for each activity, which showed the same mobility. Furthermore, when an individual gel was simultaneously stained for NAD- and NADP-dependent activities, a single band appeared (Fig. 2).

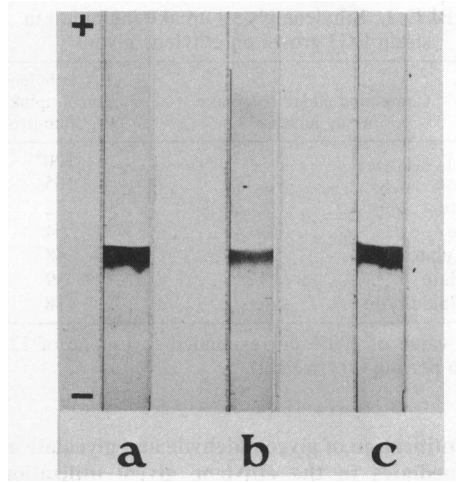

FIG. 2. Polyacrylamide gel electrophoresis of strain EG3 glycolaldehyde dehydrogenase. Extracts of strain EG3 grown on casein acid hydrolysate were run in parallel and then stained for glycolaldehyde dehydrogenase activity. The reaction mixture for activity staining contained NAD (gel a), NADP (gel b), and NAD plus NADP (gel c) as coenzyme.

Glycolate was identified as the product which resulted when glycolaldehyde dehydrogenase acted on glycolaldehyde. The reaction mixture (6 ml), which consisted of 100 mM sodium glycine buffer (pH 9.5), 3.5 mM glycolaldehyde, 5 mM NAD, and 1.5 U of partially purified glycolaldehyde dehydrogenase (DEAE chromatographed), was incubated for 30 min at 37°C. This reaction mixture was then treated as described by Sridhara and Wu (19) for the isolation of organic acids, and the product obtained was analyzed by chromatography as described above. When the chromatograms were stained with acridine, one spot with an R_f corresponding to the R_f of the glycolate standard appeared. Staining with $AgNO_3$ produced a second spot, with an R_f corresponding to the R_f of the glycine standard; this spot was evidently due to the glycine in the buffer.

Glycolate oxidase in ethylene glycol-positive mutants. Glycolate oxidase, the next enzyme in the pathway, converted glycolate into glyoxylate. Basal levels of this enzyme were low (less than 0.001 U/mg of protein) in extracts of the wild-type and mutant strains grown on casein acid hydrolysate and were induced (0.008 U/mg of protein) in extracts of strains G1, G3, and EG3 grown on glycolate. Induction was even higher (0.010 U/mg of protein) when strain EG3 was grown on ethylene glycol. The induced glycolate oxidase displayed activity in close agreement with the data described previously by other authors (8, 18).

Ethylene glycol uptake. When cells of strain G1 or G3 were grown on glycolate, their glycolate oxidase activity but not their glycolaldehyde dehydrogenase activity became highly induced. Such cells retained very little radioactivity when they were incubated with 1 mM labeled ethylene glycol. The time course showed that for these strains the rate of uptake was less than 0.3 nmol/min per mg (dry weight). On the other hand, when cells of strain EG3 were grown on glycolate or ethylene glycol, besides high induction of glycolate oxidase, glycolaldehyde dehydrogenase was also present at high levels. Such cells showed active uptake of labeled ethylene glycol, with values of 2.5 nmol/min per mg (dry weight).

Because of the combined effects of isotopic dilution and mass action, the uptake of labeled ethylene glycol by strain EG3 was inhibited by compounds of the proposed new metabolic pathway (Fig. 1). Inhibition was most pronounced with the early intermediates of the pathway. Addition of glucose up to a concentration of 25 mM to the uptake mixture caused no inhibition. Addition at the same concentration of acetate, malate, glyoxylate, glycolate, or glycolaldehyde caused a gradually increased inhibitory effect (Table 2).

TABLE 2. Ethylene glycol uptake inhibition in strain EG3 grown on ethylene glycol

Compound added to uptake assay mixture	[^{14}C]ethylene glycol uptake (% of control)
None	100[a]
Glucose	105
Acetate	72
Malate	54
Glyoxylate	48
Glycolate	39
Glycolaldehyde	18

[a] A value of 100% corresponded to uptake of 125 nmol/h per mg (dry weight).

Identification of glycolaldehyde and glycolate as intermediates in the ethylene glycol utilization pathway. The relative inhibition of uptake caused by different metabolites suggested the pathway proposed in Fig. 1, and strong support for this pathway was given by the fact that [^{14}C]glycolaldehyde and [^{14}C]glycolate derived from [^{14}C]ethylene glycol accumulated when uptake experiments were performed in the presence of high concentrations of glycolate and glyoxylate, respectively. Identification of the radioactive intermediates was performed in ethanolic extracts prepared and chromatographed as described above. Scanning of the radioactivity in chromatograms of ethanolic extracts of cells incubated with glycolate showed two peaks of radioactivity (R_f, 0.45 and 0.68), which were identified as glycolaldehyde (R_f of standard glycolaldehyde, 0.45) and ethylene glycol (R_f of standard ethylene glycol, 0.67), respectively. An additional peak of radioactivity with an R_f of 0.28 was obtained when the cells were incubated in the presence of glyoxylate, and this peak was identified as corresponding to glycolate (R_f of standard [^{14}C]glycolate, 0.27) (Fig. 3).

DISCUSSION

The strategy in selection of the pathway for ethylene glycol metabolism in *E. coli* was based on the fact that cells which were able both to convert ethylene glycol to glycolaldehyde and to utilize glycolate required only one mutation to allow them to convert glycolaldehyde into glycolate and hence to grow on ethylene glycol. This was attained in our study at an estimated frequency in the range of the frequency of a single mutation. However, the selection of strain EG3 resulted in increases in the levels of two different enzymes involved in the new metabolic pathway (propanediol oxidoreductase and glycolaldehyde dehydrogenase). At present, it is not possible to conclude whether the high levels of these two activities are the consequence of a single mutation affecting simultaneously the expression of the corresponding genes or are the result of a second mutation occurring during the growth of the mutant cells already utilizing ethylene glycol.

The conversion of ethylene glycol to glycolaldehyde, the first requirement for the parental strain, was easily achieved by propanediol oxidoreductase, which is constitutively synthesized in strains G3 and EG3. The identity of this ethylene glycol oxidative activity with propanediol oxidoreductase was ascertained by the joint phenotypic expression of propylene glycol and ethylene glycol utilization, which otherwise would be very unlikely.

The second requirement for the parental strain, utilization of glycolate, was accomplished neither by strain 1 nor by its derivative, strain 3, but, as indicated above, glycolate-positive revertants were easily selected through spontaneous mutation. According to Ornston and Ornston (18), the non-inducibility of glycolate oxidase (18) and the inducibility of malate synthase (22) by glycolate in these strains indicate a defect in glycolate oxidase or in the expression of this enzyme rather than a glycolate permeability problem.

Glycolaldehyde dehydrogenase appears to be the key enzyme in the construction of the ethylene glycol pathway. We note that strains G1 and G3, which are not utilizers of ethylene glycol, had high basal levels of activity independent of the carbon source in the culture medium. Since strain G3 showed constitutive levels of propanediol oxidoreductase, the inability of this strain to utilize ethylene glycol is related to insufficient

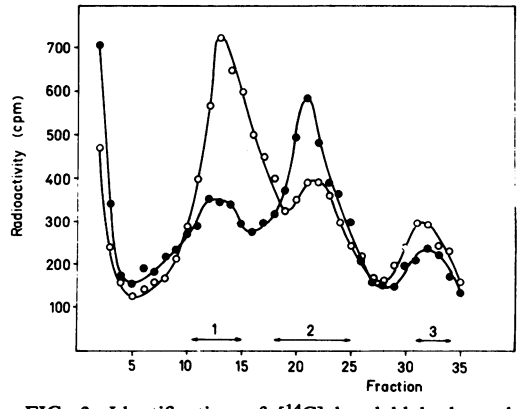

FIG. 3. Identification of [^{14}C]glycolaldehyde and [^{14}C]glycolate as intermediates in the ethylene glycol pathway: profile of the radioactivity along a chromatogram of ethanolic extracts of cells incubated with labeled ethylene glycol plus 25 mM glyoxylate (○) or 25 mM glycolate (●). The arrows indicate the positions of the [^{14}C]glycolate (arrow 1), glycolaldehyde (arrow 2), and ethylene glycol (arrow 3) standards in the chromatograms.

glycolaldehyde oxidative function. This activity was highest in strain EG3.

Although a transport system seems to be involved in the entry of propylene glycol into the cells, this compound passively diffuses quite rapidly across the phospholipid bilayer (12). Ethylene glycol may be as permeable as propylene glycol or even more so since the lack of the hydrophobic head is probably compensated for by the smaller molecular size. Actually, the strong dependence of [^{14}C]ethylene glycol uptake on its metabolism might indicate a great permeability, and presumably the only way to prevent the loss of substrate is to convert it to a charged metabolite (glycolate).

Our proposal for the pathway for ethylene glycol utilization in the cells which we used is supported by the presence of the appropriate enzymatic activities, the ability of certain compounds to inhibit ethylene glycol uptake, and the identification of glycolaldehyde and glycolate as intermediate metabolites in the pathway. This proposed pathway was constructed by a rather general mechanism in experimental evolution, which consisted of the borrowing of an enzymatic activity belonging to another pathway (5, 23). In fact, propanediol oxidoreductase, which transforms ethylene glycol into glycolaldehyde belongs to the fucose and rhamnose fermentation pathway (2, 6). Glycolaldehyde dehydrogenase, the second enzyme in the proposed pathway, is an enzyme thus far described as belonging to D-arabinose metabolism in *E. coli* (16) and in this sense may be seen as an enzyme borrowed from this pathway for ethylene glycol metabolism. LeBlanc and Mortlock indicated the possible identity of glycolaldehyde dehydrogenase with lactaldehyde dehydrogenase, another enzyme of the fucose pathway which is also responsible for utilization of D-arabinose (16).

We note that the pathway for ethylene glycol metabolism described for other bacteria isolated from natural environments (4, 10, 21) coincides with the pathway experimentally evolved in our *E. coli* cells. This seems to reinforce the evolutionary significance of experimental construction of metabolic pathways.

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