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Evolution of L-1,2-Propanediol Catabolism in *Escherichia coli* by Recruitment of Enzymes for L-Fucose and L-Lactate Metabolism

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A mutant strain of *Escherichia coli* capable of growth on L-1,2-propanediol was isolated previously. The mutant is characterized by constitutive production of a propanediol:nicotinamide adenenine dinucleotide (NAD) oxidoreductase which is essential for the new growth property. In the present study, it is shown that phage P1 cotransduces the genetic locus conferring this property and the genes for the utilization of L-fucose. A further indication of a relationship between these two growth properties is provided by the observation that wild-type *E. coli* excretes propanediol during fermentation of L-fucose. Under these conditions, a propanediol dehydrogenase (lactaldehyde reductase) is induced. This enzyme migrates on diethylaminoethyl-cellulose with the propanediol dehydrogenase produced constitutively by the mutant strain. A key event in the establishment of the ability to grow on propanediol is evidently a shift in the expression and function of propanediol dehydrogenase; an enzyme catalyzing formation of a reduced fermentation product anaerobically in wild-type cells functions aerobically to oxidize this same product in the mutant. L-Lactaldehyde, which is thus derived from propanediol, is converted to L-lactate by another dehydrogenase (L-lactaldehyde:NAD oxidoreductase) which is constitutively produced by both wild-type and mutant cells. The normal function of this enzyme is not yet established. L-Lactate is converted to pyruvate by an inducible NAD-independent L-lactate dehydrogenase. Thus, the carbons of propanediol are brought into the central metabolic network of the cell.

While studying the evolution of catabolic pathways in bacteria, Sridhara et al. (28) described the isolation of a mutant *Escherichia coli* strain that had acquired the ability to grow on L-1,2-propanediol. This ability was dependent on the appearance in the mutant of a previously undescribed enzyme, a propanediol dehydrogenase (L-1,2-propanediol:nicotinamide adenine dinucleotide (NAD) oxidoreductase) which catalyzes the following reaction:

$$\text{L-1,2-propanediol} + \text{NAD}^+ \xrightleftharpoons{\text{dehydrogenase}} \text{L-lactaldehyde} + \text{NADH} + \text{H}^+$$

The dehydrogenase was present even when the cells were grown on glucose in the absence of propanediol (28). The origin of the enzyme, however, remained a mystery. Was it an enzyme normally playing some role involving propanediol, inducible in wild-type cells only under some special conditions? Such an enzyme might have become constitutively synthesized as a result of a mutation. Alternatively, did the protein formerly act on some other compound? Such an enzyme might have expanded or modified its catalytic specificity as a result of structural alteration of the protein. The possibility, of course, also exists that both kinds of mutations have taken place during prolonged selection on the novel substrate.

Reports that *E. coli* metabolizes L-fucose (5) and L-rhamnose (25) by cleavage to lactaldehyde and that some bacteria excrete propanediol during fermentation of L-rhamnose (13) and the realization that the genetic locus which controls the ability of *E. coli* to grow on propanediol is in the chromosomal region containing the genes for the dissimilation of fucose (29) led us to search for a possible relationship between the ability to utilize and the ability to produce propanediol.

MATERIALS AND METHODS

Bacteria and phages. The parental strain 1 was *E. coli* K-12. The isolation of the mutant, strain 3, able to grow on propanediol, has been described previously

(28). Transduction with phage P1 was carried out by the method of Luria et al. (19).

Chemicals. DL-1,2-propanediol was purchased from Fisher Scientific Co., Pittsburgh, Pa., and redistilled. The sodium salt of 2,6-dichlorophenol indophenol was also from Fisher. L-Fucose, streptomycin sulfate, and NAD were obtained from Sigma Chemical Co., St. Louis, Mo; L-lactic acid was from Schwarz Bioresearch, Inc., Orangeburg, N.Y.; casein acid hydrolysate, vitamin-free, was from Nutritional Biochemicals Corp., Cleveland, Ohio; and diethylaminoethyl (DEAE)-cellulose, Whatman DE 52, was from Reeve Angel, Clifton, N.J.

Growth of cells. Carbon sources were added in a basal inorganic medium (28) in the following concentrations: hexoses, 0.01 M for aerobic growth, 0.02 M for anaerobic growth; L-lactate, 0.02 M; DL-1,2-propanediol, 0.04 M; and casein acid hydrolysate at 0.5%. Aerobic growth was carried out at 37 C in swirled Erlenmeyer flasks, 500 ml of medium in a 2-liter flask for large batches or 25 ml of medium in 300-ml sidearm flasks for small batches. Anaerobic growth on a large scale was carried out with 2 liters of medium gently stirred by a magnet in a sealed 2-liter flask equipped with stopcocks so that the gas phase could be flushed with nitrogen after the inoculation and subsequent withdrawals of samples. Anaerobic growth on a smaller scale was studied in 300-ml sidearm flasks filled to the top and stoppered. Growth was monitored with a Klett-Summerson colorimeter with a number 42 filter. One Klett unit corresponds to a cell density of 4×10^6 cells/ml. At turbidities greater than 50 Klett units, the optical readings were no longer proportional to cell densities; appropriate corrections were therefore made.

Preparation of extracts. Large batches of cells for the purpose of column chromatographic analysis of enzymatic composition were harvested by centrifugation, washed twice in a sodium phosphate buffer (0.01 M, pH 7.0), and resuspended in four times their wet weight of the same buffer. The cells were sonically disrupted in four 1-min pulses in a tube chilled at −10 C. Extracts were clarified by centrifugation for 30 min at 35,000 × *g*. Nucleic acids were removed by the addition of 0.1 volume of 20% (wt/vol) of streptomycin sulfate which had been neutralized with sodium hydroxide. The resulting suspension was stirred for 15 min and then centrifuged for 30 min at 35,000 × *g*. The absorbance at this point indicated less than 8% nucleic acid in the extracts (15). Further additions of streptomycin sulfate resulted in loss of enzyme activity.

For lactate dehydrogenase assays, 2 ml of 10^{10} cells/ml in 0.1 M sodium phosphate, pH 7.5, were sonically disrupted for two 30-s periods at 0 C.

Enzyme assays. Spectrophotometric assays for propanediol dehydrogenase and glycerol dehydrogenase were performed as described by Sridhara et al. (28), except that the final volume was 1 ml. The lactate dehydrogenase was assayed by the method of Kline and Mahler (12). The ϵ_{600} of dichloroindophenol was taken to be 16,000 (9). The concentration of the dye in the cuvette was determined by absorbance rather than weight of material added. Protein concentrations were measured with the biuret reagent (6) or, for column work, by ultraviolet absorbance (15).

Column chromatography. DEAE-cellulose was equilibrated with 0.01 M sodium phosphate buffer, pH 7.0, according to the manufacturer's instructions. A fresh column (2.3 by 15 cm) was prepared for each extract. After fivefold dilution in the phosphate buffer, the extract, containing 120 to 190 mg of protein, was put on the column, and the column was washed with 50 ml of the phosphate buffer. Proteins were then eluted with a 500-ml linear gradient of 0 to 0.6 M NaCl in the same buffer. Since the propanediol dehydrogenase eluted from the column was very unstable, enzymatic activities were measured as the fractions were collected. Sodium chloride concentration was determined from the conductivities of the samples.

RESULTS

Genetics of propanediol and fucose utilization. Interrupted mating experiments confirmed that the capacity for growth on propanediol is controlled by a locus very close to that for fucose catabolism. A connection between these two functions was further indicated by the fact that the propanediol-utilizing mutant, strain 3, lost the ability to grow on fucose both aerobically and anaerobically. Reciprocal phage P1 transduction experiments were therefore carried out with parent and mutant strains to see if the propanediol-positive trait is obligatorily associated with the fucose-negative trait. The results given in Table 1 show that, intriguingly, these two phenotypes are often but not always jointly inherited.

Since the selection of strain 3 from strain 1 passed through several mutational steps, only one of which was likely to be the constitutive synthesis of propanediol dehydrogenase, the expression of the gene for this enzyme in 35 fucose-positive transductants of strain 3 that retained their ability to grow on propanediol was analyzed by growing the cells aerobically on casein hydrolysate with or without propanediol. All of these transductants produced the enzyme constitutively at the level observed in strain 3 itself. A similar enzymatic survey was per-

TABLE 1. *Transduction of fucose and propanediol markers*[a]

Donor	Recipient	Phenotype selected	Cotransduction of unselected marker
Strain 1 (*fuc*$^+$ *prd*)	Strain 3 (*fuc prd*$^+$)	Fuc$^+$	69/170 (42%)
Strain 3 (*fuc prd*$^+$)	Strain 1 (*fuc*$^+$ *prd*)	Prd$^+$	127/140 (91%)

[a] The fucose trait was scored aerobically.

formed on 32 fucose-positive transductants of strain 3 that lost their ability to grow on propanediol. None of these synthesized significant amounts of the dehydrogenase (less than 10% observed in strain 3) even when grown on casein hydrolysate plus propanediol. Ten of these transductants were checked to see whether the addition of propanediol during exponential growth on casein hydrolysate resulted in growth inhibition. None was affected.

To find out whether the loss of the ability of strain 3 to grow on fucose is an anomaly introduced unnecessarily by multiple mutations resulting from heavy mutagenesis, a series of nine independent, spontaneous, propanediol-positive mutants were isolated from strain 1. Of these, seven lost their ability to grow on fucose both aerobically and anaerobically.

Growth on fucose and excretion of propanediol by wild-type cells. To see whether the wild-type strain from which the propanediol-positive mutant was derived excreted propanediol during growth on fucose, strain 1 was grown both anaerobically and aerobically on the methylpentose. Parallel cultures were grown on glucose as controls (Table 2). The substrate concentrations were 5 and 10 mM for aerobic cultures and 5, 10, and 20 mM for anaerobic cultures. The cellular yields were directly proportional to substrate concentration; thus, the carbon source was growth limiting. As expected, the yields of cells per mole of substrate were lower for anaerobic than aerobic growth for both carbon sources, since much of the carbohydrate must be reduced during fermentation and is not available for formation of cell materials. More significantly, the inferiority of fucose to glucose as a source of carbon and energy was more striking under anaerobic than aerobic conditions.

After stationary phase was reached, the cells were removed from each culture, and the culture fluids were analyzed for propanediol. In the bioassay of this compound, advantage was taken of the ability of strain 3 to utilize propanediol for growth. The spent fluids were sterilized by filtration, and two equal portions were inoculated with cells of strains 1 or 3. It should be borne in mind that culture media exhausted anaerobically are expected to contain fermentation products such as succinate, acetate, and lactate, which support further growth in the presence of air. However, strains 1 and 3 grow equally well on these compounds. If propanediol is also present, only strain 3 will grow on it. Hence, the yield of strain 3 minus the yield of strain 1 should be a measure of the propanediol concentration in the growth medium. These corrected yields of strain 3 on exhausted media are given in the fourth column of Table 2. In the final column, the yields are converted into propanediol concentrations.

The key results were that fucose gave rise to propanediol but glucose did not when wild-type cells were grown on the sugars, and that the amount of propanediol produced from fucose was much higher under anaerobic than under aerobic conditions. Indeed, it may be seen from Table 2 that 1 mol of propanediol was excreted per mol of methylpentose fermented. A slight amount of propanediol was also produced during aerobic growth on fucose, possibly because

TABLE 2. *Growth yield and propanediol excretion by strain 1*

Growth			Propanediol excreted into the medium	
Carbon source	Condition	Yield of strain 1[a] (Klett units/mM)	Net yield of strain 3[b] (Klett units/mM)	Calculated (L-propanediol)[c] (mM)
Glucose	Anaerobic	12.7 ± 0.2 (7)	−0.8 ± 0.2 (6)	0.0
Fucose	Anaerobic	5.0 ± 0.2 (9)	21.4 ± 0.8 (8)	1.1
Glucose	Aerobic	42.8 ± 1.2 (5)	0.0 ± 0.1 (5)	0.0
Fucose	Aerobic	33.8 ± 2.6 (6)	3.2 ± 1.2 (6)	0.2

[a] Values given are Klett units divided by the millimolarity of the carbon source, ± the standard deviation of the mean. The number of determinations is given in parentheses.

[b] Values given are the growth yield of strain 3 inoculated after the removal of cells of strain 1 in stationary phase by filtration. The yield of strain 1 on another portion of the spent medium was determined and has been subtracted, and the resulting net yield was normalized by dividing it by the millimolarity of the original medium. Number of determinations is given in parentheses.

[c] Values given were calculated from the yield of strain 3 in the fourth column of the table on the basis of 10.0 ± 0.4 Klett units of strain 3 per millimolar DL-1,2-propanediol. This was determined from 13 experiments with commercial propanediol which had been redistilled. Since only the L isomer is used for growth, one would expect 20.0 Klett units per millimolar L-propanediol.

of inadequate aeration as the population approached stationary phase.

Column chromatography of propanediol dehydrogenase. Since propanediol was produced by wild-type cells from a growth substrate giving rise to lactaldehyde, an assay for the enzyme catalyzing the interconversion of these two compounds was performed on a cellular extract of strain 1 grown anaerobically on fucose. A propanediol dehydrogenase activity was indeed detected. Anaerobically grown cells, however, are known to contain a glycerol dehydrogenase which also catalyzes dehydrogenation of propanediol. (The product in this case is acetol. The normal function of this enzyme is probably fermentative production of glycerol. The enzyme in cells grown anaerobically on glucose is present in considerably higher levels than in those grown aerobically. Further, it is present in equal quantity in cells of strains 1 and 3 [28].) To determine whether a propanediol dehydrogenase distinct from glycerol dehydrogenase had been induced, a column chromatographic study was carried out. Sonic extracts of strains 1 and 3 were prepared and subjected to DEAE column chromatography essentially as described in the previous study of the mutant enzyme (28). Strain 3 grown aerobically on propanediol, in confirmation of a previous study (28), gave the chromatographic pattern shown in Fig. 1A, with propanediol dehydrogenase being eluted first, followed by glycerol dehydrogenase. A propanediol dehydrogenase with similar elution characteristics was induced in wild-type strain 1 cells grown anaerobically on fucose (Fig. 1B); little of this enzyme was found in cells grown aerobically on fucose or anaerobically on glucose (results not shown). When an extract of strain 1 cells grown anaerobically on fucose was mixed with an extract of strain 3 cells grown aerobically on propanediol, a single component of propanediol dehydrogenase was eluted during chromatography (Fig. 1C).

Further characterization in a preliminary study of the propanediol dehydrogenases from wild-type and mutant strains by the K_m values for propanediol and glycerol indicated a difference in substrate specificity between the two proteins. Another line of evidence indicating a structural difference was provided by the more rapid heat inactivation of the enzyme from the mutant. Definitive conclusions, however, must be postponed until means are discovered to stabilize the enzymes and to reduce the variability of their properties from one preparation to another.

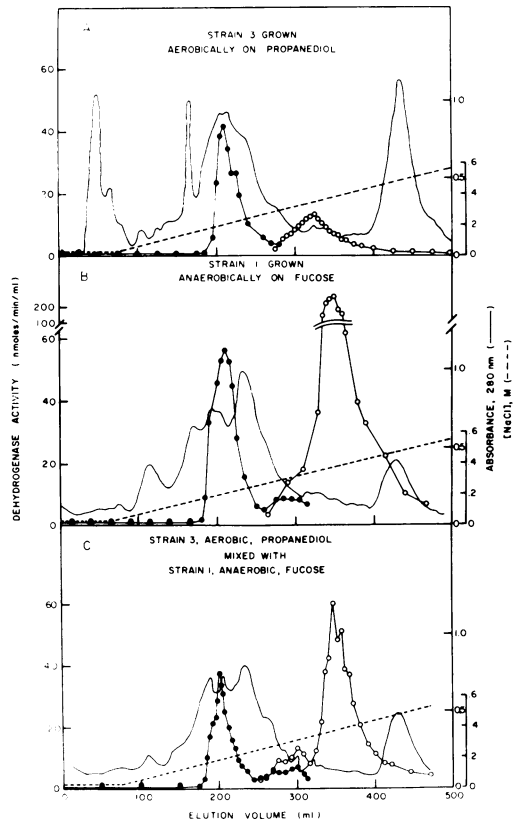

FIG. 1. *DEAE-cellulose chromatography of extracts from wild-type (strain 1) and mutant (strain 3) E. coli. Symbols:* ——, *absorbance at 280 nm;* - - - -, *molarity of sodium chloride;* ●, *propanediol dehydrogenase;* ○, *glycerol dehydrogenase. In the column represented in frame A, the usual washing after application of the sample was omitted. The first peak in the profile represents the proteins that did not absorb. These proteins were washed away in the standard procedure which was followed for the columns described by the other two frames.*

TABLE 3. *L-Lactate dehydrogenase levels in crude extracts of E. coli as a function of growth conditions*

Carbon source	Growth condition	Sp act[a]	
		Strain 1	Strain 3
Glucose	Aerobic	7	8
Glucose	Anaerobic	2	6
Lactate	Aerobic	138	108
Fucose	Aerobic	56	
Fucose	Anaerobic	4	
Propanediol	Aerobic		113

[a] Nanomoles of dichloroindophenol reduced per minute per milligram of protein.

Induction of lactate dehydrogenase. According to a previous study, the L-lactaldehyde produced from propanediol is converted to L-lactate by a constitutive NAD-dependent dehydrogenase whose usual function is not yet known for certain (27). L-Lactate thus formed is expected to be converted to pyruvate by a known enzyme that enables the cell to utilize externally provided L-lactate. To test this supposition, wild-type cells and cells of strain 3 were grown on various carbon sources and harvested during exponential growth. Sonically disrupted preparations were assayed without any centrifugation so that no activity would be eliminated in case the relevant enzyme might be membrane associated. L-Lactate dehydrogenase activity, as measured by coupling with dichloroindophenol, was present only at low levels in cells of both strains grown on glucose (Table 3). (NAD-dependent L-lactate dehydrogenase was also found in these extracts. Its specific activity did not change significantly under the various growth conditions studied. High levels of the enzyme were induced in both strains by growth on L-lactate.) Induction of the enzyme also occurred when strain 1 was grown aerobically, but not anaerobically, on fucose and when strain 3 was grown aerobically on propanediol, encouraging the belief that L-lactate is indeed an intermediate in the catabolism of propanediol.

DISCUSSION

With the exception of the nature of the permeation process, it is now possible to describe all of the biochemical reactions in the experimentally evolved pathway for the aerobic catabolism of L-propanediol by *E. coli*. Acting successively, propanediol dehydrogenase and lactaldehyde dehydrogenase convert the compound to L-lactate, which is finally transformed to pyruvate by an inducible NAD-independent lactate dehydrogenase (probably of the flavoprotein class). The pathways by which pyruvate can be phosphorylated and thence used as the sole source of cellular carbon and energy have been described by Cooper and Kornberg (2). Figure 2 summarizes this view of propanediol catabolism. It also outlines the catabolic pathway of fucose in the wild-type *E. coli*. Half of the fucose gives rise to lactaldehyde. Under anaerobic conditions, the lactaldehyde is reduced to propanediol and excreted. Aerobically, the lactaldehyde can be dehydrogenated to lactate, possibly by the enzyme catalyzing the same reaction in the propanediol pathway of strain 3.

It would appear that during the course of selection the lactaldehyde reductase functioning during anaerobic growth of wild-type cells on fucose changed into the enzyme that catalyzes the reverse reaction as a dehydrogenase during aerobic growth on propanediol of mutant cells. The exact number and nature of mutations that are required for this evolution of function are by no means clear. Quite possibly, both the primary structure of the lactaldehyde reductase and the expression of its gene have

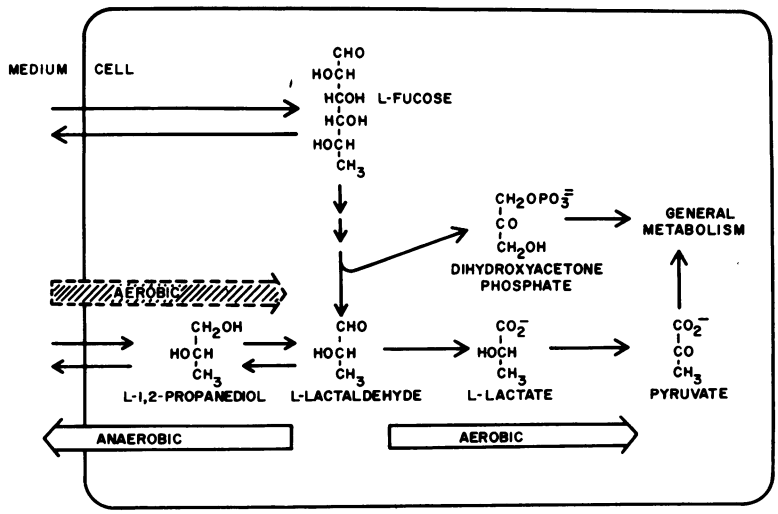

FIG. 2. *Proposed scheme for the aerobic catabolism of propanediol by mutant E. coli (large hatched arrow) and for the aerobic and anaerobic catabolism of L-fucose by the wild type. The initial products of L-fucose catabolism are L-fuculose and L-fuculose-1-phosphate* (5).

been altered. A single mutation to constitutivity cannot account for all of the properties of strain 3. The relationship between the growth ability on propanediol and the loss of the ability to grow on fucose still remains in the dark both biochemically and genetically and thus instigates further investigation. Nonetheless, the basic findings in this study provide another, though more intricate, example of what is emerging as a general strategy employed by nature in developing new catabolic pathways. The regulation of a key enzyme or permease is altered so that it can be present under the selective conditions. The novel carbon source is thereby transformed to a compound that the cell is equipped to handle via ordinary pathways (1, 3, 4, 7, 8, 10, 11, 14, 16-18, 20-24, 26, 30-32). But here a new twist appears—the net flow of the reaction catalyzed by the enzyme is reversed, and an anaerobic enzyme is converted to an aerobic one.

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