JOURNAL OF BACTERIOLOGY, June 1976, p. 1166-1172
Vol. 126, No. 3

Printed in U.S.A.

Disruption of the Fucose Pathway as a Consequence of Genetic Adaptation to Propanediol as a Carbon Source in *Escherichia coli*

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Received for publication 2 December 1975

In *Escherichia coli*, L-fucose is dissimilated via an inducible pathway mediated by L-fucose permease, L-fucose isomerase, L-fuculose kinase, and L-fuculose 1-phosphate aldolase. The last enzyme cleaves the six-carbon substrate into dihydroxyacetone phosphate and L-lactaldehyde. Aerobically, lactaldehyde is oxidized to L-lactate by a nicotinamide adenine dinucleotide (NAD)-linked dehydrogenase. Anaerobically, lactaldehyde is reduced by an NADH-coupled reductase to L-1,2-propanediol, which is lost into the medium irretrievably, even when oxygen is subsequently introduced. Propanediol excretion is thus the end result of a dismutation that permits further anaerobic metabolism of dihydroxyacetone phosphate. A mutant selected for its ability to grow aerobically on propanediol as a carbon and energy source was reported to produce lactaldehyde reductase constitutively and at high levels, even aerobically. Under the new situation, this enzyme serves as a propanediol dehydrogenase. It was also reported that the mutant had lost the ability to grow on fucose. In the present study, it is shown that in wild-type cells the full synthesis of lactaldehyde dehydrogenase requires the presence of both molecular oxygen and a small molecule effector, and the full synthesis of lactaldehyde reductase requires anaerobiosis and the presence of a small molecule effector. The failure of mutant cells to grow on fucose reflects the impairment of a regulatory element in the fucose system that prevents the induction of the permease, the isomerase, and the kinase. The aldolase, on the other hand, is constitutively synthesized. Three independent fucose-utilizing revertants of the mutant all produce the permease, the isomerase, the kinase, as well as the aldolase, constitutively. These strains grow less well than the parental mutant on propanediol.

Strain 3, a mutant of *Escherichia coli* strain 1 which had acquired the ability to grow on L-1,2-propanediol as the sole source of carbon and energy, produces constitutively a propanediol:nicotinamide adenine dinucleotide (NAD) oxidoreductase, which converts the substrate to L-lactaldehyde. L-Lactaldehyde is further metabolized to L-lactate under the influence of another NAD-linked oxidoreductase produced by both wild-type and mutant cells (11). L-Lactate then induces internally yet another dehydrogenase, but of the flavo-protein class, which converts the substrate to pyruvate (2), thus feeding the carbons of propanediol into the central metabolic network.

A connection between the metabolism of propanediol and fucose was first suggested by the observation that the genetic locus that confers the new catabolic capability can be cotransduced by phage P1 with genes for the utilization of fucose. It was also found that wild-type strain 1 growing anaerobically on fucose excretes propanediol and exhibits induced levels of a propanediol:NAD oxidoreductase. This enzyme migrated together with the constitutive enzyme of the mutant during column chromatography on diethylaminoethyl-cellulose. In addition, it was noticed that strain 3 no longer grows on fucose, either aerobically or anaerobically (2).

L-Fucose is metabolized in *E. coli* (Fig. 1) by the sequential action of L-fucose isomerase (6), L-fuculose kinase (7), and L-fuculose 1-phosphate aldolase (4). The products of the last reaction are dihydroxyacetone phosphate and L-lactaldehyde. Evidently, under anaerobic conditions, all of the lactaldehyde is reduced to propanediol and excreted into the medium, but under aerobic conditions the aldehyde is fully catabolized by oxidation (2). In strain 3, the propanediol:NAD oxidoreductase is produced constitutively not only anaerobically, but aero-

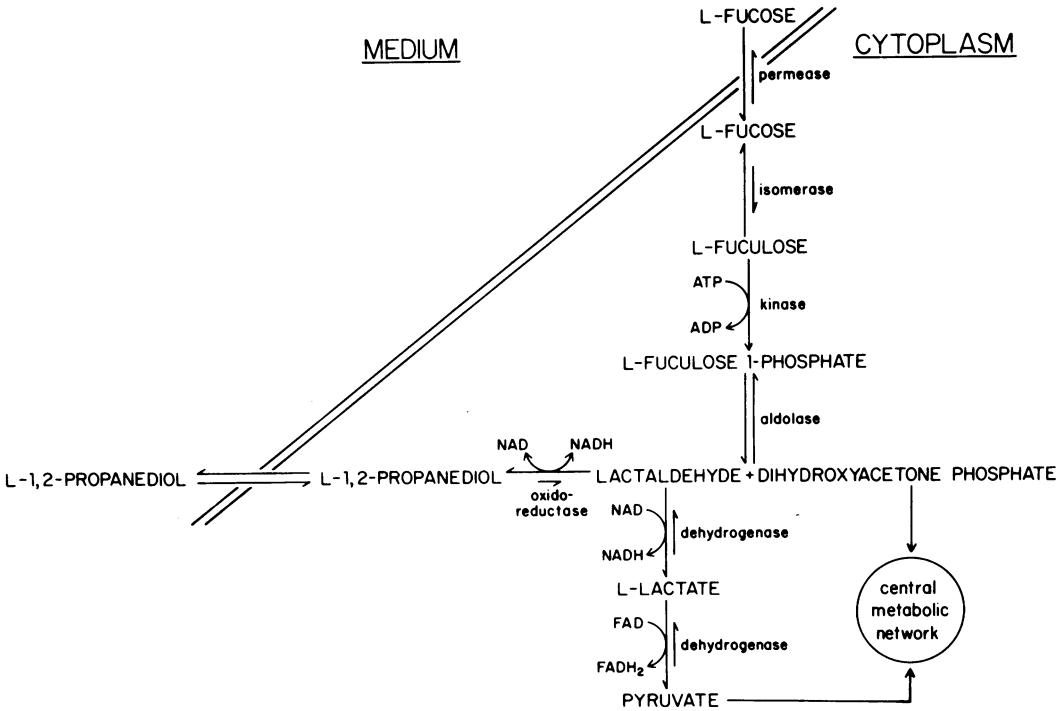

FIG. 1. *Scheme for propanediol and fucose metabolism in E. coli. The enzyme catalyzing the interconversion of propanediol and lactaldehyde is referred to as propanediol oxidoreductase in this study, instead of propanediol dehydrogenase (which had been used previously), since the actual role of this protein depends upon the strain in which it is found. ATP, Adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; FAD, flavin adenine dinucleotide.*

bically as well, a necessary regulatory change permitting the reversal of the physiological role of this enzyme from a reductase to a dehydrogenase.

The primary objectives of the present study have been to characterize the biochemical basis for the inability of strain 3 to grow on fucose and to determine whether or not the L-lactaldehyde dehydrogenase described previously as a constitutive enzyme indeed belongs to the fucose system by a more careful examination of its levels in strains 1 and 3 grown under various physiological conditions.

MATERIALS AND METHODS

Chemicals. L-Fuculose was obtained by using a mutant strain of *Klebsiella aerogenes* that excretes this compound into the medium when supplied with L-fucose (E. St. Martin, Ph.D. thesis, Univ. of Massachusetts, Amherst, 1975). Fuculose production was monitored by the cysteine-carbazole method for detection of keto sugars (3). When production of fuculose was maximal, the medium was passed through membrane filters (0.45-μm pore diameter; Millipore Corp., Bedford, Mass.), and the filtrates were used without further treatment.

L-Fuculose 1-phosphate was prepared by an enzymatic procedure (7) with the following modifications. A strain of *K. aerogenes* that lacked fuculose 1-phosphate aldolase (St. Martin, Ph.D. thesis) was used as source of fuculose kinase. Cell extracts were used without further purification. After deproteinization and removal of nucleotides by adsorption on activated charcoal, the solution was concentrated to 50 ml by rotary evaporation and was applied to a column (40 by 1.5 cm) of AG1-X10 resin (50 to 100 mesh, formate form; Bio-Rad Laboratories, Richmond, Calif.), which had been equilibrated with water. The column was washed with 50 ml of water, and a linear gradient of 0 to 1 M sodium formate and 0.4 M formic acid in a total volume of 800 ml was applied. The effluent was collected in 10-ml fractions and was assayed for cysteine-carbazole-reacting material. Free fuculose was eluted in the void volume. Fractions containing fuculose 1-phosphate were pooled and brought to pH 6.4 with 0.3 M barium hydroxide, and 4 volumes of ethanol were added. The precipitate collected by centrifugation after overnight storage at 0 C was washed in 80% ethanol and redissolved in 10 mM acetic acid.

L-Lactaldehyde was prepared by the reaction of ninhydrin with D-threonine (13). Since preparations thus obtained caused, at high concentrations, inhibition of L-lactaldehyde dehydrogenase, further purification was carried out by precipitation and washing the product with absolute ethanol and collecting

the precipitated lactaldehyde dimer by centrifugation. The dimers were cleaved by heating the preparation at 80 C in 0.1 M HCl for 10 min (8), and the preparation was characterized by thin-layer chromatography in ethyl acetate-acetic acid-water (3:1:3, vol/vol/vol) and methanol-NH_4OH-water (6:1:3 vol/vol/vol) (13). The concentration of lactaldehyde was estimated by bisulfite binding (1).

DL-1,2-Propanediol was purchased from Fisher Scientific Co., Pittsburgh, Pa., and redistilled. L-Fucose was obtained from Pfanstiehl Laboratories, Inc., Waukegan, Ill.; L-[1-^{14}C]fucose was from Amersham/Searle, Arlington Heights, Ill.; casein acid hydrolysate (vitamin free) was from Nutritional Biochemicals Corp., Cleveland, Ohio; and purified enzymes were from Boehringer Mannheim Biochemicals, New York, N.Y.

Bacteria. Strain 3, able to grow on propanediol, but no longer able to grow on fucose, was isolated from *E. coli* K-12 strain 1 (12). Independent revertants of strain 3, capable of growth on fucose, strains 54, 55, and 56, were selected on 10 mM fucose minimal agar. Colonies that appeared after several days of incubation at 37 C were suspended in 10 ml of sterile saline, and single cells were replated on fucose minimal agar.

Growth of cells. Carbon sources were added to a basal inorganic medium (12) in the following concentrations (either alone or as mixtures): hexoses, 0.01 M for aerobic growth, 0.02 M for anaerobic growth; pyruvate, 0.02 M; DL-1,2-propanediol, 0.04 M; and casein acid hydrolysate, 0.5%. Other growth conditions were described before (2).

Preparation of extracts. Cells were harvested in mid- to late exponential phase by centrifugation, washed in 0.1 M sodium phosphate buffer (pH 7.0), and suspended in four times their wet weight in the same buffer containing 2.5 mM glutathione. The cells were sonically disrupted by two 1-min pulses (20 kcycles/s) in a tube chilled at 0 C. The supernatant fluids were used for enzyme assays after centrifugation at 100,000 × *g* for 45 min at 0 C (to remove NADH oxidase).

Enzyme assays. L-Fucose isomerase activity was determined from the rate of L-fuculose formation by the cysteine-carbazole method (3). Reaction mixtures (1.0 ml) contained L-fucose (10 μmol), tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at pH 7.0 (20 μmol), and $MnCl_2$ (2 μmol). Assays were started by the addition of bacterial extract (50 to 100 μg of protein). The blank mixture lacked L-fucose.

The assay of L-fuculose kinase (EC 2.7.1.51) was based on the fuculose-stimulated production of adenosine 5′-diphosphate in a coupled system with pyruvate kinase and lactate dehydrogenase (7). Reaction mixtures (1 ml) contained Tris-hydrochloride buffer at pH 7.8 (50 μmol), $MgCl_2$ (5 μmol), adenosine 5′-triphosphate (10 μmol), phosphoenolpyruvate (2.5 μmol), glutathione (1 μmol), L-fuculose (2 μmol), NADH (0.25 μmol), NaF (5 μmol), lactate dehydrogenase (50 μg), and pyruvate kinase (50 μg). The blank mixture lacked L-fuculose. Assays were started by the addition of bacterial extract (50 to 100 μg of protein) and changes in the extinction coefficient at 340 nm (E_{340}) were recorded.

The assay of L-fuculose 1-phosphate aldolase (EC 4.1.2.17) was dependent upon the formation of dihydroxyacetone phosphate (4).

Propanediol oxidoreductase (EC 1.1.1.55) (12) and lactaldehyde dehydrogenase (EC 1.2.1.22) (11) activities were measured spectrophotometrically by the formation of NADH in a final total volume of 1 ml. Propanediol oxidoreductase was also measured by using L-lactaldehyde as substrate when high levels of glycerol dehydrogenase were expected to be present in extracts. This latter enzyme catalyzes the interconversion of propanediol and acetol but not of propanediol and lactaldehyde (12). Assay mixtures (1 ml) contained sodium carbonate buffer (pH 9.5; 100 μmol), L-lactaldehyde (5 μmol), and $NADH_2$ (0.15 μmol). The blank reaction mixture lacked L-lactaldehyde. Assays were started by the addition of bacterial extract (50 to 100 μg of protein), and changes in E_{340} were recorded.

All reaction rates were measured in a Gilford recording spectrophotometer. Protein concentrations were measured with the biuret reagent (5).

Transport assay. L-Fucose permease activity was determined by the rate of L-[1-^{14}C]fucose uptake by whole cells. Cells were harvested in the late exponential phase of growth, washed with mineral medium, and suspended at approximately 10^9 cells per ml of mineral medium containing chloramphenicol (100 mg/ml). The assay was started by adding 0.5 ml of the cell suspension to 0.5 ml of mineral medium containing L-[1-^{14}C]fucose (0.2 μmol, 0.16 μCi). Samples (0.1 ml) were withdrawn at 30-s intervals, and the uptake reaction was terminated by delivery onto a membrane filter (0.45 μm) previously wetted with mineral medium. The filter was immediately washed with 5 ml of mineral medium and dried, and the radioactivity was determined by scintillation counting. Uptake of radioactivity by the cells was found to be linear over a 4-min period.

RESULTS

Fucose enzymes in the wild-type organism. In cells of strain 1 growing on fucose as the sole source of carbon and energy, or on fucose plus casein hydrolysate, the specific activities of fucose permease, fucose isomerase, fuculose kinase, and fuculose 1-phosphate aldolase were induced to 10-fold greater levels than those in cells grown on casein hydrolysate. Induction was independent of the aeration of the cultures (Table 1).

Propanediol oxidoreductase was most highly induced in cells grown on fucose anaerobically. Intermediate levels were found in cells grown on fucose aerobically with or without casein hydrolysate and in cells grown on casein hydrolysate whether aerobically or anaerobically. A very low level of this enzyme was found in cells grown on glucose aerobically. However, assay of this enzyme in cells grown on glucose anaerobically requires special caution because, in a previous study, cells grown anaerobically on glucose and fumarate were shown to contain

TABLE 1. *Activities of fucose pathway enzymes in crude extracts of wild-type E. coli as a function of growth conditions*

Carbon source	O_2	Sp act[a]					
		Fucose permease[b]	Fucose isomerase	Fuculose kinase	Fuculose 1-phosphate aldolase	Propanediol oxidoreductase	Lactaldehyde dehydrogenase
CAA[c]	+	0	36	27	0	20	93
CAA + fucose	+	55	500	440	240	80	100
Fucose	+	58	520	420	130	42	280
Glucose	+					6	15
CAA + pyruvate	−					89	0
Fucose	−	64	750	310	270	240[d]	32
Glucose	−					120[e]	0

[a] Nanomoles per minute per milligram of protein at 25 C.
[b] Nanomoles per minute per milligram of dry weight at 37 C.
[c] Casein hydrolysate.
[d] Measurement in reverse by lactaldehyde reduction gave 360 units.
[e] Measurement in reverse by lactaldehyde reduction gave zero activity. See text.

a high level of glycerol dehydrogenase, which also attacks propanediol, but at C2 (12). By measuring both the dehydrogenation of propanediol and the reduction of lactaldehyde, it was shown in the present study that cells grown anaerobically on glucose alone did contain a significant level of glycerol dehydrogenase but did not contain a detectable level of propanediol oxidoreductase. We therefore conclude that full induction of propanediol enzyme requires both the presence of an inducer and anaerobiosis and that glucose exerts a strong repression both aerobically and anaerobically.

Lactaldehyde dehydrogenase was most highly induced in cells grown aerobically on fucose as the sole source of carbon. During anaerobic growth on fucose or aerobic growth on fucose in the presence of casein hydrolysate, only partial induction was evident. The enzyme was strongly repressed in cells grown anaerobically on casein hydrolysate plus pyruvate, and on glucose whether O_2 was present or not.

Fucose enzymes in strain 3. The lack of induction of fucose permease, fucose isomerase, and fuculose kinase evidently accounts for the inability of strain 3 to grow on fucose since their basal activities were not elevated in cells grown on casein hydrolysate in the presence of the substrate. On the other hand, fuculose 1-phosphate aldolase, with no apparent function in cells blocked in the initial portion of the pathway, was found to be synthesized at high constitutive levels (Table 2).

Propanediol oxidoreductase, as expected from the results of early studies, was produced at high constitutive levels, especially under aerobic conditions. Its synthesis, however, was repressed during growth on glucose, apparently more so in aerobic cultures, but not to the very low level observed in strain 1.

Lactaldehyde dehydrogenase was most highly induced in cells grown aerobically on propanediol. During aerobic growth on casein hydrolysate in the presence or absence of fucose, only partial induction was evident. The enzyme was strongly repressed in cells grown on casein hydrolysate plus pyruvate anaerobically or on glucose alone whether O_2 was present or not.

Fucose revertants of strain 3. Fucose-positive revertants occur at a frequency of about 5×10^{-7} among cells of strain 3 (J. Aguilar, unpublished data). Growth on fucose of three such independent revertants, strains 54, 55, and 56, in each case was slower than that of strain 1. On the other hand, growth of these revertants on propanediol was slower than that of strain 3, although the differences were slight. A competitive growth experiment on propanediol between cells of strain 3 and those of revertant 56 (with the fastest growth rate, both on fucose and on propanediol) was therefore carried out to ascertain whether the gain of one function had to be at some expense of the other. Cells of strain 56 (1.4×10^8) and cells of strain 3 (1.1×10^8) were inoculated into 500 ml of propanediol mineral medium. When the population reached a density of about 10^9 cells per ml, 0.1 ml of the culture was used to inoculate 250 ml of fresh propanediol medium. After two further similar transfers (about 45 generations in total), an analysis revealed that fucose-negative cells (strain 3) outnumbered fucose-positive cells (revertant 56) by more than 100:1.

An enzymatic examination was then carried out to determine the basis of the restored ability of strains 54, 55, and 56 to grow on fucose. Fucose permease, fucose isomerase, fuculose kinase, and fuculose 1-phosphate aldolase were all found to be synthesized constitutively (Ta-

TABLE 2. *Activities of fucose pathway enzymes in crude extracts of strain 3 as a function of growth conditions*

Carbon source	O_2	Sp act[a]					
		Fucose permease[b]	Fucose isomerase	Fuculose kinase	Fuculose 1-phosphate aldolase	Propanediol oxidoreductase	Lactaldehyde dehydrogenase
CAA[c]	+	0	43	80	490	410	130
CAA + fucose	+	0.08	30	46	490	390	110
Propanediol	+		43	57	490	420	260
Glucose	+					50	36
CAA + pyruvate	−					260	9
Glucose	−					92	7

[a] Nanomoles per minute per milligram of protein.
[b] Nanomoles per minute per milligram of dry weight.
[c] Casein acid hydrolysate.

TABLE 3. *Activities of fucose pathway enzymes in crude extracts of revertants of strain 3 grown aerobically*

Strain no.	Carbon source	Sp act[a]			
		Fucose permease[b]	Fucose isomerase	Fuculose kinase	Fuculose 1-phosphate aldolase
54	CAA[c]	0.3	110	110	470
	CAA + fucose	0.3	100	120	500
55	CAA	8.0	230	160	500
	CAA + fucose	7.0	240	160	440
56	CAA	15.0	600	550	370
	CAA + fucose	15.0	630	400	420

[a] Nanomoles per minute per milligram of protein.
[b] Nanomoles per minute per milligram of dry weight.
[c] Casein acid hydrolysate.

ble 3). In no case did the recovery of the fucose permease activity approach the levels found in the wild-type strain. The isomerase and the kinase specific activities were similar within each revertant, although they differed between revertants. The specific activities of the aldolase remain similar to that in strain 3. The levels to which the permease, the isomerase, and the kinase were restored correspond in order to the growth rates of the revertants on fucose (Table 4).

DISCUSSION

The pathway of fucose dissimilation diverges at the aldolase step, which yields dihydroxyacetone phosphate and lactaldehyde. Dihydroxyacetone phosphate is destined to supply the glycolytic pool irrespective of the redox condition. The fate of lactaldehyde, however, is critically dependent upon the aeration of the cells.

Under anaerobic conditions, propanediol oxidoreductase (acting as lactaldehyde reductase) is fully induced, and the propanediol formed by this enzyme is excreted into the medium. The disposal of two hydrogens in this manner allows the further metabolism of dihydroxyacetone phosphate without the need for an exogenous hydrogen acceptor. For each mole of fucose consumed anaerobically, approximately 1 mol of propanediol is released into the medium (2).

Under aerobic conditions, lactaldehyde dehydrogenase is fully induced, and the lactate formed by this enzyme is oxidized to pyruvate by an inducible dehydrogenase (2). The availability of molecular oxygen thus obviates the need of internal dismutation and permits all the carbons of fucose to be consumed.

TABLE 4. *Aerobic growth rates of wild-type, mutant, and revertant cells on fucose and propanediol*

Strain no.	Doubling time (min) on:	
	Fucose (10 mM)	Propanediol (10 mM)
1	90	−[a]
3	−[a]	140
54	420	170
55	270	170
56	110	160

[a] No growth.

A previous observation that a derivative of strain 3 missing lactaldehyde dehydrogenase activity can no longer grow on propanediol (11) and the demonstration in the present study that this enzyme is inducible aerobically by propanediol in strain 3 and is inducible aerobically by fucose in the wild-type strain 1 lead us to conclude that lactaldehyde is indeed the physiological substrate. There is no evidence pointing to a genetic alteration of the control of lactaldehyde dehydrogenase synthesis in strain 3. The observation that aerobic growth of this mutant on propanediol results in the induction of this enzyme to a level close to that in the wild-type strain grown aerobically on fucose as the sole source of carbon and energy suggests that an intermediate in the fucose pathway, possibly lactaldehyde itself, is the inducer. In the wild-type strain, the provision of casein hydrolysate diminishes or obscures the inducing effect of fucose, possibly by decreasing the intracellular concentration of the inducer derived from fucose.

It was reported that the ability of strain 3 to grow aerobically on propanediol at a rate approaching that of aerobic growth on glycerol required several mutational steps (12). It has since been shown that some early-stage mutants were able to grow on propanediol without noticeable impairment of the capacity to grow on fucose. Continued selection of propanediol, however, favors mutants that have a block in the fucose pathway (J. Aguilar and A. Hacking, unpublished data).

Two possibilities can be suggested for the failure of strain 3 to synthesize fucose permease, fucose isomerase, and fuculose kinase: (i) a change in a common regulatory mechanism for the three enzymes; (ii) the structural conversion of fucose permease to a propanediol transport protein, resulting in either the inability of the inducer to enter the cell or a severe polar mutation preventing expression of the genes coding for the isomerase and kinase. Under the second alternative, one would expect the fucose-positive revertants of strain 3 either to regain inducibility of that pathway or become fully constitutive in it. In fact, two out of three revertants were partially constitutive in the synthesis of the isomerase and kinase. A comparison of the levels of fucose permease activity in cells of strain 1 and cells of revertant strains 55 and 56 grown at 30 and 42 C (but assayed under standard conditions) did not uncover any evidence for altered thermal stability.

If a specific transport system were required to overcome poor permeability of the cell membrane to propanediol, a prime candidate would be the system required to facilitate the exit of propanediol during normal fermentation of fucose. If the control of this system is not linked to that for propanediol oxidoreductase, a constitutive mutation should suffice.

In the case of glycerol transport by *E. coli*, loss of the protein capable of catalyzing the facilitated diffusion of the substrate prevented normal growth only when the concentration of glycerol in the medium fell below 5 mM (9). The half-time of equilibration of 400 mM glycerol across such permeation-defective cells was approximately 18 s, as determined by an optical assay based on plasmolysis (10). The propanediol permeability of wild-type cells grown even under conditions not causing the induction of the anaerobic fucose pathway was too rapid to be recorded by this method (half-time less than 2 s) (J. Aguilar and F. E. Ruch, unpublished data).

On the basis of these observations, we therefore favor the regulatory model at present, but further experimental confirmation is required.

It is not clear whether the derepression of fuculose 1-phosphate aldolase is mechanistically connected with the non-inducibility of the other proteins of the fucose system or is the partial and indirect result of another genetic change leading to improved propanediol utilization.

It is becoming increasingly evident that the construction of an effective metabolic pathway involves considerably more than introducing and increasing the desired biochemical activities through the duplication and/or modification of genes coding for certain catabolic proteins. Proper balance of the rate of production of each intermediate must be assured to avoid both growth-restricting bottlenecks and toxic accumulation of metabolites. The process may be further complicated by the necessity of remodeling other cellular components not directly involved in the evolving system, but which happen to share with it, for instance, regulatory mechanisms or common protein subunits. Finally, wasteful synthesis of the proteins of the new pathway must be minimized by the establishment of new controls. Numerous kinds of mutations may therefore be essential before a developing system can be harmoniously integrated within the organism. The evolving propanediol system may prove to be a productive and challenging model to study the coadaptation of genes.

ACKNOWLEDGMENTS

We are grateful to Edwart St. Martin and Tai Te Wu for helpful advice, and to Juan Aguilar for the use of strains 54, 55, and 56, which he isolated.

This investigation was supported by grant GB-43288X from the National Science Foundation and by Public Health Service grant 5 RO1 GM11983 from the National Institute of General Medical Sciences.

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