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Derepression of an NAD-Linked Dehydrogenase That Serves an *Escherichia coli* Mutant for Growth on Glycerol

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An *Escherichia coli* mutant using an NAD-linked dehydrogenase instead of an ATP-dependent kinase as the first enzyme for glycerol dissimilation excreted dihydroxyacetone during the initial phase of growth. The intermediate was salvaged as growth of the culture advanced. The transient loss of the intermediate into the medium appeared to be partly determined by variation of the level of glycerol dehydrogenase with growth conditions. With up to 2% casein hydrolysate as the carbon and energy source, the cellular level of the dehydrogenase increased 1 order of magnitude at the end of growth. This increase was probably caused by the depletion of certain metabolites and was prevented by the addition of pyruvate or glucose to the growth medium. The repressive effect of these compounds was not lifted by the addition of cyclic AMP. Diminution of oxygen tension in the culture medium with increased cell density was not directly responsible for the increase of the enzyme level. Thus, neither catabolite repression nor respiratory repression was implicated as an important control mechanism in the synthesis of this enzyme. Since increases in the specific activity of the enzyme in cell extracts reflected increases in the concentration of the enzyme protein, post-translational control was also not involved. A novel kind of regulation of gene expression is indicated.

Glycerol dissimilation in *Escherichia coli* is normally mediated by proteins coded by the *glp* system. The compound entering via a facilitator protein that catalyzes substrate equilibration across the cytoplasmic membrane is trapped by an ATP-dependent kinase. The product, *sn*-glycerol 3-phosphate, is then converted to dihydroxyacetone phosphate by one of the two flavin-linked dehydrogenases, according to the nature of the terminal electron acceptor available (8). A multistep mutant, strain 424, was isolated in which the innate glycerol pathway (rendered inactive by abolishment of glycerol kinase and aerobic *sn*-glycerol 3-phosphate dehydrogenase) is replaced by a new pathway with an NAD^+-linked dehydrogenase as the first enzyme (14). This enzyme reversibly converts glycerol to dihydroxyacetone (DHA). The protein was purified to electrophoretic homogeneity and was shown to have a broad substrate specificity (16). The synthesis of this enzyme in the mutant appeared at first to be constitutive. However, during the course of a quantitative study of glycerol utilization by the mutant, we were led to the discovery that the level of the enzyme is highly dependent upon the physiological state of the cell. In this report, we describe some critical growth conditions that influence the enzyme level.

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MATERIALS AND METHODS

Chemicals. Morpholinepropanesulfonic acid, glutardialdehyde (grade I), alkaline phosphatase (type VII), lysozyme, *p*-nitrophenylphosphate, human serum albumin, Tween 20, glycerol dehydrogenase, L-lactate dehydrogenase, pyruvate kinase, and glycerol kinase were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were purchased as previously described (16) or were of reagent grade. Disposable polystyrene tubes (10 by 55 mm) were obtained from VWR Scientific Co.

Bacterial strains and growth conditions. *E. coli* strain 424 was derived from strain 204 as a transductant that grew on agar containing 10 mM glycerol as the sole carbon and energy source (14). Strain 204, a K-12 derivative, is a mutant lacking both glycerol kinase and aerobic glycerol 3-phosphate dehydrogenase (11). A medium containing 0.1 M phosphate was used for growth (15), unless a low phosphate concentration was required to minimize the decomposition of DHA, in which case a morpholinepropanesulfonic acid-buffered

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medium with the following composition was used: 1 mM potassium phosphate, 40 mM KCl, 34 mM NaCl, 20 mM $(NH_4)_2SO_4$, 1 μM $FeSO_4$, 3 mM $MgSO_4$, 1 μM $ZnCl_2$, 10 μM $CaCl_2$, and 75 mM morpholinepropanesulfonic acid at pH 7.5. Unless otherwise specified, cells were grown in 250-ml cultures incubated at 37°C in 2-liter Erlenmeyer flasks agitated at approximately 240 rpm on rotary shakers. Culture density was monitored by reading in a Klett colorimeter (no. 42 filter). One Klett unit is equivalent to 4×10^6 cells per ml.

Determination of glycerol and DHA content in culture media. Glycerol content was determined enzymatically by following the appearance of NADH in an assay mixture also containing glycerol kinase, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate, and ATP (3). DHA content was determined enzymatically by the disappearance of NADH catalyzed by glycerol dehydrogenase in 1 ml of an assay mixture containing 0.015 U of glycerol dehydrogenase, 0.2 mM NADH, 10 mM NH_4Cl, and 80 mM potassium phosphate at pH 7.0. In each case a linear standard curve was obtained.

Assay and purification of glycerol dehydrogenase. Glycerol dehydrogenase was assayed and purified to homogeneity from *E. coli* strain 424 as previously described (16). Units of enzyme activity are expressed in micromoles per minute per milligram of protein.

Preparation of antibodies against the enzyme. Specific antisera against the dehydrogenase were raised in rabbits immunized with the purified enzyme, and antibody titers were determined by an enzyme neutralization assay (13). The specific antibody levels were about 150 U/ml.

Preparation of labeled glycerol dehydrogenase for enzyme-linked immunosorbent assay. The conjugation of purified *E. coli* glycerol dehydrogenase to alkaline phosphatase was based on a method of Engvall et al. (1). A phosphatase preparation (2.3 mg of protein) suspended in 2.6 M ammonium sulfate was collected as a pellet by centrifugation. After the supernatant fraction was discarded, 0.45 ml of 50 mM phosphate buffer (pH 7.0) containing 0.75 mg of the dehydrogenase was added to resuspend the phosphatase. The mixture was dialyzed overnight at 4°C against phosphate-buffered saline (0.9% sodium chloride in 15 mM sodium phosphate at pH 7.2). Glutardialdehyde was added at a final concentration of 0.2%. The mixture was incubated for 2 h at 20°C, diluted to 1 ml with phosphate-buffered saline, and dialyzed again overnight against phosphate-buffered saline. The content was then chromatographed on a Sepharose 6B column (1.5 by 90 cm) in 0.05 M Tris-hydrochloride buffer (pH 8.0). The conjugated dehydrogenase was eluted from the column with the void volume and stored at 4°C in the presence of 5% human serum albumin and 0.02% sodium azide. The preparation was stable under these conditions for at least 6 months. The phosphatase activity of the conjugate was about 120 optical density units at 400 nm per min per ml when assayed at 20°C in a reaction mixture containing 1 mg of *p*-nitrophenylphosphate per ml, 1 mM $MgCl_2$, and 50 mM sodium carbonate buffer at pH 9.8.

Quantitative determination of glycerol dehydrogenase by the enzyme-linked immunosorbent assay method. The determination of glycerol dehydrogenase as an antigen was also based on the procedure of Engvall et al. (1). One milliliter of 0.1 M sodium carbonate buffer (pH 9.8) containing 2 μg of immunoglobulin G from specific antisera against glycerol dehydrogenase was added to each of a series of polystyrene tubes. After incubation for 3 h at 37°C, the tubes were stored at 4°C with the antibody solution.

Before use, the antibody-coated tubes were washed three times with a solution of 0.05% Tween 20 and 0.9% NaCl. Unknown samples or standard amounts of glycerol dehydrogenase (0 to 250 ng) dissolved in 0.5 ml of phosphate-buffered saline containing 1% human serum albumin and 0.02% sodium azide were then added to a series of the tubes. Each tube then received 0.1 ml of diluted alkaline phosphatase-conjugated glycerol dehydrogenase in the same phosphate-buffered saline solution (0.02 optical density units at 400 nm per min). The tubes were incubated for 15 h at 20°C and then washed three times with a solution of 0.9% NaCl plus 0.05% Tween 20. The amount of enzyme-linked antigen that was bound to the antibody-coated tubes was determined by measuring the activity of alkaline phosphatase with *p*-nitrophenylphosphate as a substrate.

RESULTS

Growth of strain 424 on glycerol and DHA. For wild-type *E. coli* K-12 employing its native pathway, glycerol at a concentration below 10 μM becomes growth rate limiting (5). In contrast, for mutants lacking glycerol facilitator, but still possessing glycerol kinase, a substrate concentration below 10 mM becomes growth rate limiting (11). Strain 424 appeared not only to have retained the glycerol facilitator, but also to synthesize it constitutively (the half-time of equilibration of glycerol across the cytoplasmic membrane of cells grown on casein hydrolysate was less than 1 s by an optical osmotic method [6]). However, when strain 424 was tested for growth on glycerol, the generation time increased progressively as the substrate concentration descended below 10 mM (data not shown). It is therefore likely that the activity of the enzyme in the cell (the apparent K_m for glycerol is 1.4 mM in 100 mM NH_4^+ as an activator [16] and is more than 1 order of magnitude higher in the absence of a monovalent cation that serves as an activator [8]), rather than substrate permeation, determined the rate of growth.

In another experiment, the cellular level of the dehydrogenase and the rate of substrate consumption were followed during the course of growth in a medium initially supplied with 20 mM glycerol as the source of carbon and energy. Since the immediate product, DHA, is a small and uncharged compound which might be difficult for the plasma membrane to retain effectively, the possibility of excretion of the intermediate was also tested. Cells from a culture grown to stationary phase on glycerol were used to inoculate a fresh medium. Figure 1 shows that during the initial phase of growth, the specific

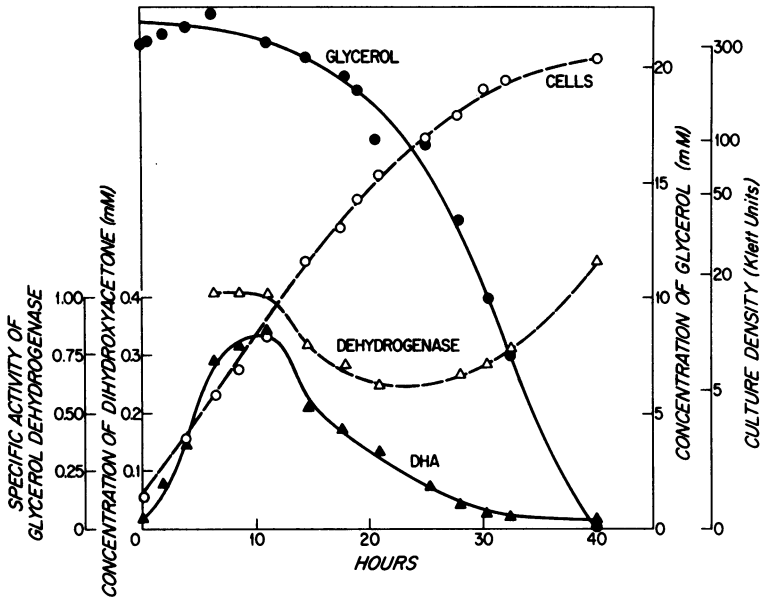

FIG. 1. Transient accumulation of DHA in the medium during growth on glycerol. Overnight glycerol-grown cells were inoculated into a series of flasks containing fresh medium (20 mM glycerol in morpholinepropanesulfonic acid medium). Growth was monitored by periodically withdrawing samples. One or more cultures were pooled and harvested at chosen time intervals by membrane filtration (filters from Millipore Corp., Bedford, Mass.). The filtrate was assayed enzymatically for glycerol and DHA concentrations. The collected cells were resuspended at a density of 500 Klett units in mineral medium. After mixing with 9% chloroform for 5 min and subsequent incubation with lysozyme (125 μg/ml) at 30°C for 10 min, the debris was removed by centrifugation. The supernatant fraction was used for measuring glycerol dehydrogenase activity.

activity of the dehydrogenase was maintained at a fairly constant level. During this period, there was net excretion of DHA into the medium which exceeded 0.3 mM at its peak. Salvaging of the excreted compound commenced after 10 h; at this time the dehydrogenase also began to decrease, suggesting that DHA excretion was the result of excessive glycerol dehydrogenation (a specific activity of about 1 apparently exceeded the capacity of DHA utilization). As the glycerol concentration in the medium descended below 15 mM, the level of the dehydrogenase rose again. However, the salvaging of the excreted DHA continued. This would suggest that the effect of the increase in the enzyme level was offset by the reduction of specific enzyme activity in vivo as a result of decreased substrate saturation. An increase in the capacity of DHA utilization is not a likely explanation, since the growth rate was already decelerating.

The disappearance of DHA from the medium was not the result of spontaneous decomposition because media supplied with this compound as the sole source of carbon and energy supported final cell yields similar to those observed with glycerol. When the two compounds were supplied together, the yields were additive (Table 1).

Change in the specific activity of glycerol dehydrogenase during growth on casein hydrolysate. Fluctuation of the dehydrogenase level with growth phase was found to occur also with other carbon sources. Indeed, drastic changes were observed with casein acid hydrolysate. When cells grown fully on 2% of this carbon source were inoculated at a low population density into a fresh medium, a 20-fold fall of enzyme level occurred during the first 3 h as growth resumed (Fig. 2). The total enzyme units in the culture, however, did not decrease. As growth approached the stationary phase, the high enzyme level was restored (see below).

Growth in the presence of spent medium. To test for excretion of a stimulating factor by cells during terminal growth on 2% casein hydroly-

TABLE 1. Growth on glycerol and dihydroxyacetone

Carbon source (g/liter)		Final cell yield (Klett units)[a]
Glycerol	Dihydroxyacetone	
0.5	0	120
1.0	0	210
0	0.5	89
0	1.0	200
1.0	1.0	400

[a] The cells were grown in morpholinepropanesulfonic acid medium.

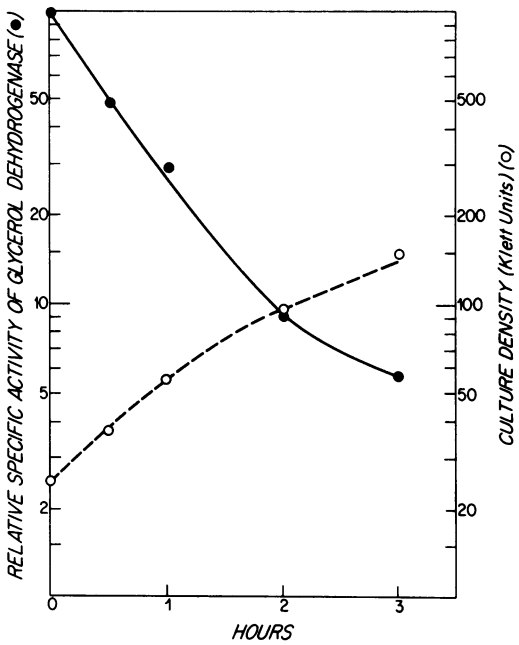

FIG. 2. Decrease in specific glycerol dehydrogenase activity during renewed growth of a cell population transferred from a stationary-phase culture. A culture grown overnight in mineral medium supplemented with 2% casein hydrolysate was diluted 30 times into fresh medium, and samples were withdrawn after 0.5, 1, 2, and 3 h. Extracts of the cells were assayed for glycerol dehydrogenase activity. Full activity represents 1.4 U/mg of protein.

sate, the spent medium was recovered, mixed with different proportions of fresh mineral medium, replenished with 2% casein hydrolysate, and reinoculated with cells. The cultures were all harvested at the same density during early exponential growth. The cells grown in mixtures containing between 0 to 77% of spent medium showed no significant difference in glycerol dehydrogenase levels.

Effect of surface/volume ratio of cultures on enzyme level. To test whether the steep rise in glycerol dehydrogenase level in cells approaching the end of growth on casein hydrolysate was caused by decreasing environmental oxygen tension, five cultures of increasing volumes were incubated in flasks of the same size on a rotary shaker and harvested at the same density for measurement of enzyme activity. A progressive increase in specific enzyme activity with decreasing surface/volume ratio of the culture was found (Fig. 3). However, as revealed by the experiment described below, this increase in glycerol dehydrogenase level was not directly attributable to the lowering of oxygen tension. Rather, inefficient aeration seemed to deplete more rapidly certain nutrients in the medium, with a repressive effect on enzyme synthesis.

Effect of nutrient concentration on the enzyme level. To distinguish a direct respiratory effect of oxygen tension from a secondary effect of the depletion rate of certain nutrients, cultures with increasing concentrations of casein hydrolysate as the carbon and energy source were harvested for measurement of enzyme activity at 190 Klett units, the maximal growth afforded by the medium containing the lowest concentration of carbon source. A rapid increase in enzyme level occurred only in the culture initially supplied with the least amount of casein hydrolysate (Fig. 4). Because all of the cultures were harvested at the same density, oxygen tension in the medium was not the likely variable. If there was a difference in oxygen tension, the culture with the lowest concentration of casein hydrolysate would be expected to have the highest oxygen tension, since the growth rate (and therefore the rate of oxygen consumption) should be the first to diminish. To find out whether the low dehydrogenase level was maintained during early exponential growth by contaminating compounds that were gradually utilized (commercial casein hydrolysate contains less than 60% by weight as amino acids), another growth experiment was carried out with media containing different concentrations of a synthetic amino acid mixture resembling casein hydrolysate in composition (3). Again, elevation of the enzyme level first occurred in the culture initially containing the lowest concentration of the carbon and energy source.

The inference that the exhaustion of certain metabolites permitted enzyme derepression was supported by the ability of 5 mM pyruvate or

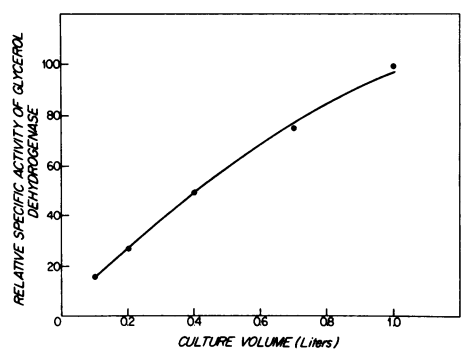

FIG. 3. Relative specific glycerol dehydrogenase activity in cells grown under different conditions of aeration. The cells were grown in mineral medium supplemented with 2% casein hydrolysate. The cultures, varying from 0.1 to 1 liter, were incubated in 2-liter flasks on a rotary shaker operated at 190 cycles per min. Each culture was harvested at 190 Klett units, and cell extracts were assayed for specific glycerol dehydrogenase activity. Full activity represents 1 U/mg of protein.

glucose (but not glycerol) to prevent the rise in dehydrogenase level in cells growing on 2% casein hydrolysate. This repressive effect was not countered by 5 mM external cyclic AMP. Furthermore, cells grown anaerobically on 2% casein hydrolysate plus 5 mM pyruvate (to stimulate anaerobic growth) or on 0.2% glucose alone (glycerol alone did not support anaerobic growth) contained low enzyme levels, again showing that anaerobiosis per se was not responsible for the increase in the dehydrogenase level.

Effect of growth temperature. The level of the dehydrogenase was also influenced by growth temperature. The data in Fig. 5 shows a threefold increase in the specific enzyme activity with the elevation of growth temperature from 30 to 39°C. For this comparison, all the cells were grown on 2% casein hydrolysate and harvested at a culture density of 190 Klett units.

Correlation of enzyme activity with protein concentration. Finally, the possibility that the acute rise in enzyme activity during terminal growth on casein hydrolysate reflected a modification of the dehydrogenase rather than a change in its concentration was tested by comparing the specific activity of the enzyme in various cell extracts with the concentration of immunochemically cross-reacting material. A series of cultures inoculated at low cell density were harvested at different stages of growth for the measurements. The increase in enzyme activity closely paralleled the amount of antigen (Fig. 6).

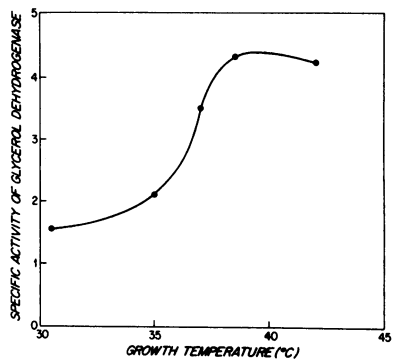

FIG. 5. Effect of growth temperature on the specific activity of glycerol dehydrogenase. The cells were grown on 2% casein hydrolysate at the various temperatures indicated and harvested when the culture density reached 190 Klett units. The cell extracts were assayed for glycerol dehydrogenase activity.

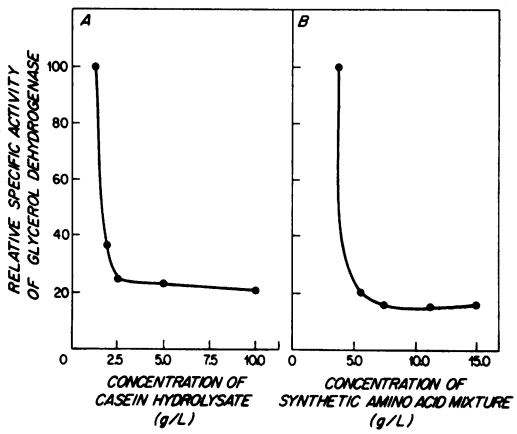

FIG. 4. Levels of glycerol dehydrogenase activity in cells harvested at the same culture density from media initially containing different concentrations of casein amino acids. A, The cells were grown in media containing increasing concentrations of casein hydrolysate. A concentration of 0.125% when fully utilized gave a culture density of 190 Klett units. All of the cultures were harvested at this density, and extracts of the cells were assayed for specific glycerol dehydrogenase activity. Full activity represents 0.93 U/mg of protein. B, The cells were grown in media containing increasing concentrations of a synthetic amino acid mixture mimicking the composition of casein hydrolysate. A concentration of 0.37% of this mixture when fully utilized gave a culture density of 190 Klett units. All of the cultures were harvested at this density, and extracts of the cells were assayed for specific glycerol dehydrogenase activity. Full activity represents 1.4 U/mg of protein.

DISCUSSION

The activity of an NAD-linked dehydrogenase serving *Klebsiella pneumoniae* naturally for glycerol dissimilation also increases radically in the cells as aerobic growth on the compound

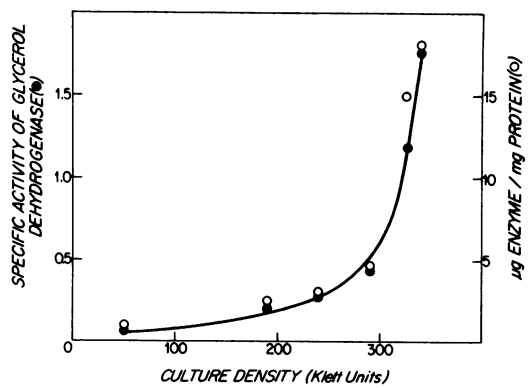

FIG. 6. Correspondence of glycerol dehydrogenase specific activity with immunochemically cross-reacting material in extracts of cells harvested at different culture densities. Cells were inoculated at less than 1 Klett unit into medium containing 2% casein acid hydrolysate. Extracts of cells harvested at different densities were assayed for enzyme activity and immunochemically by the enzyme-linked immunosorbent assay.

approaches stationary phase, but when the stationary-phase cells are allowed to regrow at low density in fresh medium, the activity of the enzyme decreases much more rapidly than can be accounted for by dilution of the protein through growth. An energy-dependent aerobic inactivation process and cessation of further gene expression are jointly responsible for this loss of enzyme activity. The inactivation of the enzyme is not paralleled by disappearance of the immunochemically cross-reacting material (9, 13).

The glycerol dehydrogenase of *K. pneumoniae* is a member of the inducible *dha* system whose function is for anaerobic utilization of the substrate. Under such a condition, this system has a crucial advantage over the *glp* system in that no exogenous hydrogen acceptor is required. This is made possible by an ancillary branch of the *dha* system that converts glycerol to 3-hydroxypropionaldehyde, which is then reduced by NADH to trimethylene glycol. NAD is thus regenerated, and the glycol is excreted into the medium.

Under aerobic conditions, however, the *glp* system is preferable to the *dha* system because of superior scavenging power. The inequality of this power reflects the large difference in the substrate K_m between glycerol kinase (10 μM) and glycerol dehydrogenase (2 mM) (2, 7, 10). The difference in the kinetic properties in turn can be understood within the framework of the Haldane relationship (4), which stipulates that a low K_m can evolve without sacrifice of V_{max} only when the equilibrium of the reaction catalyzed is sufficiently in favor of the products. The phosphorylation of glycerol at the expense of ATP is essentially irreversible. By contrast, the dehydrogenation of glycerol at the expense of NAD is very unfavorable at neutral pH.

Aside from the limitation of scavenging power attainable by glycerol dehydrogenase, there might be another handicap: the product, DHA, might be difficult for the cell to retain because the compound is a small, uncharged molecule. Moreover, some of the DHA that is retained can spontaneously give rise to the highly toxic methylglyoxal (12).

A combination of undesirable features of the *dha* system under aerobic conditions might therefore be responsible for the evolution in *K. pneumoniae* 1033 of two different mechanisms for disencumbering itself of glycerol dehydrogenase as soon as the *glp* system becomes operative.

The variation of glycerol dehydrogenase activity with growth phase in the *E. coli* mutant only superficially resembles the case of the inducible glycerol dehydrogenase of *K. pneumoniae* 1033. First, the enzyme activity in *E. coli* strain 424 varies proportionally with the immunochemically cross-reacting material, and the total enzyme activity in the culture does not diminish after the shift from anaerobiosis to aerobiosis. Hence, there is no post-translational mechanism for controlling the activity of the enzyme. Second, the apparent reduction in the expression of the gene cannot be explained by respiratory repression; synthesis of the protein seems to be regulated by the intracellular level of a specific metabolite(s). The increase in enzyme synthesis in cells growing to the stationary phase on casein hydrolysate and the prevention of this increase by pyruvate or glucose suggest that the derepression occurs with the exhaustion of amino acids, giving rise to a common metabolite that can also come from glycolysis or gluconeogenesis. A more precise notion of the nature of this metabolite might come from the eventual identification of the true function of the enzyme in wild-type cells. Finally, the failure of cyclic AMP to overcome this repression renders catabolite repression as an unlikely control mechanism.

It is somewhat surprising that three successive selections for increased basal synthesis of the enzyme have not sufficed to liberate the gene from specific regulation. This would suggest that gene duplication is more likely to be responsible than modification of the promoter region in amplifying the basal expression. The mechanism of this regulation in wild-type cells and the nature of genetic changes in the mutants present a challenge for future investigation.

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