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Glycerol Assimilation by a Mutant of *Rhodopseudomonas capsulata*

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A spontaneous mutant of *Rhodopseudomonas capsulata*, capable of growth on glycerol, has been isolated. The mutant requires CO_2 or malate to assimilate glycerol photosynthetically. This requirement is not manifested aerobically. Glycerokinase (EC 2.7.1.30) and pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase (EC 1.1.2.1) activities appear coincidently with the metabolism of glycerol, suggesting that this organism employs these enzymes for glycerol dissimilation.

The photosynthetic bacteria satisfy their immediate energy requirements by adjusting their content of energy-converting membrane in response to specific environmental changes. Furthermore, the ease with which this "adjustment" can be experimentally manipulated renders these organisms ideal for the study of membrane biogenesis and differentiation. The nonsulfur purple photosynthetic bacterium, *Rhodopseudomonas capsulata*, is particularly useful in this type of research due to its rapid growth rate and ability to grow both photosynthetically and aerobically. Studies have been conducted on the enzymatic and photopigment changes that accompany environmentally induced alterations in the membrane complex of this organism (12, 13). In addition, evidence has been presented for a differentiation in phospholipid composition between cytoplasmic and "additional" membrane formed by *R. capsulata* (22). Unfortunately, examination of this organism's lipid metabolism has been hindered by its inability to assimilate glycerol (23). This report describes the isolation of a mutant (L_1) of *R. capsulata* which possesses the ability to utilize glycerol under both aerobic (dark) and anaerobic (light) conditions of growth.

Two glycerol dissimilatory pathways are known to occur in prokaryotic organisms (10). One pathway is mediated by a glycerokinase (EC 2.7.1.30) and a pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase (EC 1.1.2.1) (5), whereas a second pathway is mediated by a nicotinamide adenine dinucleotide-dependent glycerol dehydrogenase (EC 1.1.1.6) and a dihydroxyacetone kinase (1, 15). The regulatory properties of these two pathways suggest that the former functions primarily during aerobic metabolism, whereas the latter is associated predominately with an anaerobic mode of growth (10, 15).

In our studies, strain L_1 was found to possess the enzymes comprising the aerobic route of glycerol dissimilation. Furthermore, this route, as in *Escherichia coli* (9), appears to function for both aerobic and anaerobic glycerol dissimilation. Under photosynthetic conditions, strain L_1 was found to possess levels of glycerokinase (EC 2.7.1.30) and pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase (EC 1.1.2.1) activities significantly higher than those present in the parental strain. The presence of these enzymes, concomitant with the ability of this organism to utilize glycerol, strongly suggests that this organism is similar to a number of others in employing the aerobic pathway for glycerol dissimilation.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The organism used in this study was derived from the nonsulfur purple photosynthetic bacterium, *R. capsulata* (ATCC no. 23782), by an enrichment procedure. The selection, isolation, and subsequent culture of this mutant (designated L_1) were achieved by using a malate glycerol-minimal salts medium. This medium (initial pH, 6.8) contained: 0.1% $(NH_4)_2SO_4$, 0.0001% thiamine hydrochloride, 0.5% (54 mM) glycerol, a growth-limiting amount of DL-malic acid 0.12% (9 mM), and additional inorganic salts as specified by Sojka et al. (20). Except where specified, the medium employed for the growth of the parental organism contained 0.4% (30 mM) DL-malic acid, 0.1% $(NH_4)_2SO_4$, 0.0001% thiamine hydrochloride, and additional inorganic salts as previously described (20).

Milk-dilution bottles (125-ml capacity) and 1-liter Roux bottles were employed for the photosynthetic growth of L_1 and wild-type *R. capsulata*. A photosynthetically growing culture in the logarithmic growth

phase was the inoculum source. All photosynthetic incubations were at 30 C with continuous saturating illumination (550 ft-c) provided by Lumiline lamps. Anaerobiosis was obtained by completely filling the culture vessel with medium or by incubating under an atmosphere of oxygen-free gas. Aerobic cultures were grown in 500-ml Erlenmeyer flasks containing 250 ml of media. All aerobic incubations were at 30 C in the dark. Aeration was provided by shaking. Growth rates were determined from serial turbidity measurements of the culture by using a Klett-Summerson photometer equipped with a no. 66 filter.

Preparation of cell-extracts. The cells were harvested in logarithmic phase by centrifugation and were freed of residual medium by washing in 0.05 M KH_2PO_4 buffer. The cells were resuspended in phosphate buffer (pH 7.0) and disrupted by passage through a French pressure cell (16,000 lb/in^2). The resulting extract was freed of whole cells and debris by centrifugation at 5,100 $\times$ *g*, and the crude supernatant fluid obtained was centrifuged at 106,000 $\times$ *g* for 75 min. The supernatant fluid from this latter step was decanted and the pellet was resuspended in 0.05 M phosphate buffer.

Bacteriochlorophyll and protein estimations. In the initial characterization of L_1 (Fig. 3), bacteriochlorophyll (BCHL) estimations were made by the method of Cohen-Bazire et al. (4) on 5-ml culture samples. The pelleted cells were resuspended in 0.1 ml of water and the BCHL was extracted with 4.9 ml of acetone-methanol (7:2 vol/vol). Absorbancy of the extract at 775 nm was measured and BCHL per ml of culture was calculated by using an extinction coefficient of 75 mM^{-1} cm^{-1} (3). All other BCHL estimations were made in vitro by employing the bovine serum albumin (BSA) method of Sojka et al. (19).

Protein was estimated by the method of Lowry et al. (16) by employing crystalline BSA as the standard. For the estimation of whole cell protein, the cells were harvested and the pellet was treated with 1 N NaOH for 5 min in a boiling-water bath. Samples of the resulting solution were then assayed for protein by the Lowry method.

Enzyme assays. Glycerokinase activity was measured by coupling the formation of L-α-glycerophosphate with its oxidation to dihydroxyacetone-phosphate in the presence of commercial pyridine nucleotide-linked L-α-glycerophosphate dehydrogenase (2). The reaction mixture (pH 9.5) contained, in a final volume of 1 ml, the following: adenosine 5'-triphosphate (ATP), 10 μmol; $MgCl_2$, 10 μmol; hydrazine sulfate, 0.15 mmol; nicotinamide adenine dinucleotide (NAD), 1.0 μmol; L-α-glycerophosphate dehydrogenase, 0.10 mg; glycylglycine buffer (pH 9.5), 250 μmol; and the indicated amount of glycerol and soluble, cell-free extract. The reaction was run at 25 C and was initiated by the addition of substrate. The absorbance at 340 nm was read against a reference cuvette lacking glycerophosphate dehydrogenase. Initial velocities were corrected for a slight endogenous rate, and specific activities are expressed as nanomoles of glycerophosphate formed per minute per milligram of protein.

The activity of the pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase was measured by following the glycerophosphate-dependent reduction of the tetrazolium dye, 3(4,5-dimethylthiazolyl-1,2)2,5-diphenyl tetrazolium bromide (MTT), to its formazan by the method of Lin et al. (14). The reduction of MTT was monitored by following the increase in absorbance at 550 nm. The complete reaction mixture contained, in a final volume of 1 ml, the following components: MTT, 0.048 μmol; phenazine methosulfate (PMS), 0.10 mg; KCN, 7.5 μmol; phosphate buffer (pH 7.5), 75 μmol; DL-α-glycerophosphate (GP) (calculated for the L-isomer); and particulate, cell-free extract. The reaction was run at 25 C and was initiated by the addition of substrate. Substrate was omitted in the reference cuvette. Specific activity is expressed as nanomoles of MTT reduced per minute per milligram of protein.

Identification of the products of the glycerokinase reaction. To verify that glycerophosphate was being produced, the reaction products were analyzed by paper chromatography and autoradiography. A reaction mixture containing: ATP, 5.0 μmol; $MgCl_2$, 5.0 μmol; ^{14}C-glycerol (sp act 0.10 μCi/μmol), 5.1 μmol; glycylglycine (pH 9.5), 100 μmol; and cell-free extract (final volume 0.5 ml) was incubated at 25 C. The reaction was initiated by the addition of labeled substrate and was allowed to proceed for 20 min, at which time the reaction was terminated by boiling for 5 min. To facilitate precipitation, the mixture was placed in an ice bath prior to clarification by centrifugation. The clarified supernatant fluid was cochromatographed on Whatman no. 1 filter paper in two different solvent systems along with authentic ^{14}C-glycerol and L-[^{14}C]α-glycerophosphate. An acidic solvent system containing ethyl acetate-acetic acid-water (3:3:1 vol/vol/vol) was used for the development of one chromatogram, whereas another was developed in a basic system composed of methyl Cellosolve-methyl ethyl ketone-3 N NH_4OH (7:2:3 vol/vol/vol) (7). Both chromatograms were run at 25 C. After their development, the chromatograms were dried and the radioactive areas were located by autoradiography.

Chemicals. Phenazine methosulfate, disodium DL-α-glycerophosphate, MTT tetrazolium, L-α-glycerophosphate dehydrogenase (rabbit muscle), NAD, 30% BSA, and DL-malic acid were purchased from the Sigma Chemical Co. Uniformly labeled ^{14}C-glycerol was obtained from the Amersham/Searle Corp. L-[^{14}C]α-glycerophosphate (uniformly labeled) was obtained from the International Chemical and Nuclear Corp.

RESULTS

Repeated experiments employing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as a mutagen failed to produce an organism with a glycerol-utilizing phenotype. Isolation was finally accomplished by a technique similar to the one described for the selection of an arsenate-resistant mutant of *R. capsulata* (24). A minimal salts medium containing 54 mM glycerol and a

growth-limiting amount (9 mM) of DL-malate was inoculated with photosynthetically grown, logarithmic-phase cells. This culture was incubated anaerobically in the light. Control experiments revealed that 9 mM malate could support a culture density of 440 μg (dry weight) of cells per ml of culture. Growth occurred normally to this density, whereupon turbidity increase ceased. After a 24-h stationary period, turbidity increase resumed at an extremely slow rate and continued to increase for 10 days. The final turbidity was twice that observed at the point corresponding to the exhaustion of malate, and the additional turbidity was attributed to the selection of a spontaneous mutant capable of glycerol assimilation. Repeated daily transfer of the culture through the malate-glycerol medium resulted in a substantial increase in growth rate and final cell yield. A mutant was finally isolated from the terminal enrichment culture by agar shake dilution, and the isolate was designated L_1. L_1 retained the characteristic morphology, vitamin requirements, and absorption spectrum (Fig. 1) of the parent strain. The mutant displayed a biphasic pattern of growth on the malate-glycerol medium; however, no diauxie was observed. The increase in cell number, dry weight, protein, and BCHL that occurred after the point of malate depletion indicates that the turbidity increases noted cannot simply be attributed to accumulation of cellular storage products (Fig. 2).

Although the lengthy time period required for the initial glycerol adaption indicated the selection of a spontaneous mutant, the possibility that a physiological adaptation had occurred still existed. This possibility was eliminated by allowing the organism to complete 12 mass doublings in a malate medium minus glycerol. Subsequent incubation in the malate-glycerol medium resulted in L_1 retaining its ability to grow on glycerol (no lag) after the point corresponding to the depletion of available malate.

All attempts to grow L_1 photosynthetically with glycerol as the sole source of carbon have been unsuccessful. There is an obligate requirement for malate, or a subsequent catabolite, for the assimilation of this compound. This "malate dependence" is best exemplified by Fig. 3. In this plot, glycerol assimilation, as measured by maximum cell yield, is shown to be proportional to the amount of malate present in the medium. The high Y-intercept observed is a result of residual malate or a malate catabolite present in the inoculum. The same experiment conducted in the absence of glycerol (Fig. 3) indicates the amount of growth expected from the complete utilization of malate. Both D- and L-malate are known to support the growth of *R. capsulata* (21).

The stimulatory effect of malate upon glycerol utilization is much greater than stoichiometry would predict for a direct coupling of malate utilization to the assimilation of glycerol. From the stoichiometry indicated in Fig. 3, it ap-

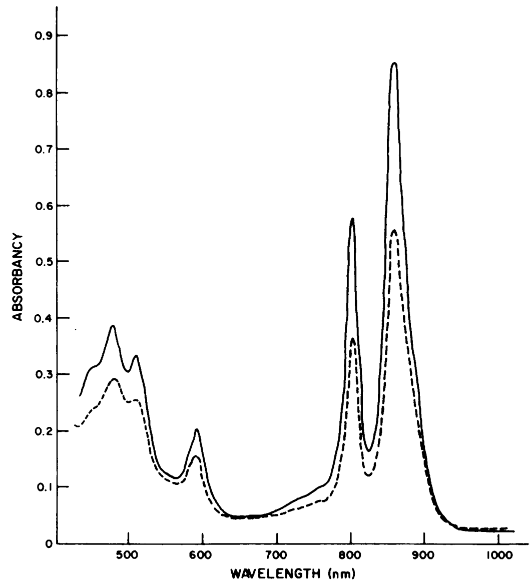

FIG. 1. *Absorption spectra of wild type and strain L_1. Spectra were determined in 25% bovine serum albumin (19). Wild type (---); L_1 (——).*

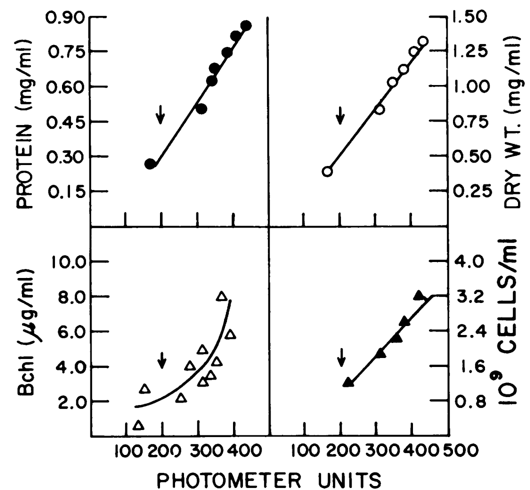

FIG. 2. *Increases in protein (16), dry weight, bacteriochlorophyll (3, 4), and cell number of L_1 after the point of malate depletion in a malate-glycerol medium. The arrow indicates the maximum amount of growth obtainable with the malate present.*

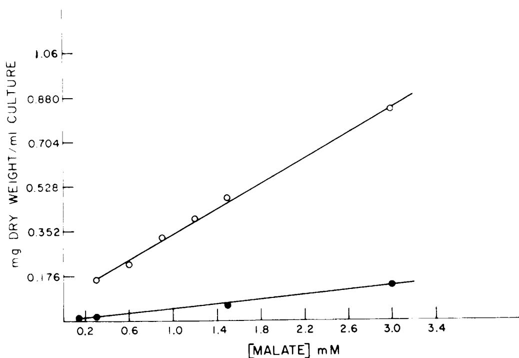

FIG. 3. *Maximum cell yield (photosynthetic) of L_1 as a function of the malate concentration. Glycerol absent (●); 54 mM glycerol (O).*

pears that reducing equivalents produced from glycerol catabolism are not accepted directly by malate. Since the photometabolism of oxidized substrates such as malate are known to give rise to a net production of CO_2 (17), the actual role of malate may be one of supplier of CO_2 for the production of dicarboxylic acids. A requirement of this type is not surprising since the photosynthetic bacteria have been shown to require the addition of CO_2 or acetate to their culture medium to metabolize carbon compounds that are more reduced than carbohydrates (6, 17). As expected, replacement of malate with bicarbonate or gaseous CO_2 permitted photosynthetic utilization of glycerol by L_1 (Table 1).

Unlike the photosynthetic situation, dark (aerobic) conditions, without any CO_2 source other than the atmosphere, support growth of L_1 on glycerol. The parent strain remained incapable of significant growth on glycerol under similar conditions (Table 2).

Attempts to elucidate the pathways of the observed glycerol assimilation revealed that L_1 possessed the enzyme activities comprising the aerobic route for glycerol dissimilation (10). Cell-free extracts prepared from cultures of L_1 grown photosynthetically on malate-glycerol media exhibited glycerokinase and pyridine nucleotide-independent glycerophosphate dehydrogenase activities at levels significantly higher than those observed in the parent strain under similar conditions.

Glycerokinase activity was localized in the soluble fraction of the cell extract (80% recoverable in the 106,000 $\times$ g supernatant fluid) and displayed a sp act of 250 nmol per min per mg of protein. As shown in Fig. 4, initial rates were dependent upon the concentration of extract protein (0.01–0.10 mg of protein/ml) and, by employing the coupled assay, absolute requirements for the presence of ATP, NAD, and commercial L-α-GP dehydrogenase in the incubation mixture were observed (Table 3). Although comparable rates were obtained in the presence and absence of hydrazine, the removal of this trapping agent markedly shortened the linear portion of the reaction. The reduction of NAD in the absence of glycerol (Table 3) was minimized by extensive dialysis of the extract. An apparent K_m for glycerol of 27 μM was determined in the dialyzed preparation (Fig. 5).

The product of the glycerokinase reaction was separated from the remaining reaction components and identified by paper chromatography and autoradiography, as described in Materials and Methods. The results obtained by using this procedure demonstrated that the complete incubation mixture contained a compound that cochromatographed with authentic L-[^{14}C]α-GP in both solvent systems. This compound was absent in those incubations in which either ATP or cellular extract was omitted.

Cell-free extracts of L_1 also contained an enzyme capable of mediating the oxidation of GP. This activity, localized (71%) in the par-

TABLE 1. *Cell yields from the photosynthetic growth of wild type and L_1 on glycerol, glycerol-CO_2, malate, and malate-glycerol media*[a]

Strain	Carbon source			
	Glycerol[b]	Glycerol-CO_2[c]	Malate[d]	Malate-glycerol[e]
Wild type	17	17	1,144	440
L_1	38	480	1,210	1,628

[a] The values are expressed as micrograms (dry weight) of culture per milliliter.
[b] Glycerol, 54 mM.
[c] Glycerol (54 mM) and gaseous carbon dioxide.
[d] DL-malate, 30 mM.
[e] Glycerol, 54 mM; malate, 9 mM.

TABLE 2. *Cell yields from dark aerobic growth of wild type and L_1 on glycerol, malate, and glycerol-malate media*[a]

Strain	Carbon source		
	Glycerol[b]	Malate[c]	Glycerol-malate[d]
Wild Type	35	733	260
L_1	742	742	1,232

[a] The values are expressed as micrograms (dry weight) of culture per milliliter.
[b] Glycerol, 54 mM.
[c] DL-malate, 30 mM.
[d] Glycerol, 54 mM; DL-malate, 9 mM.

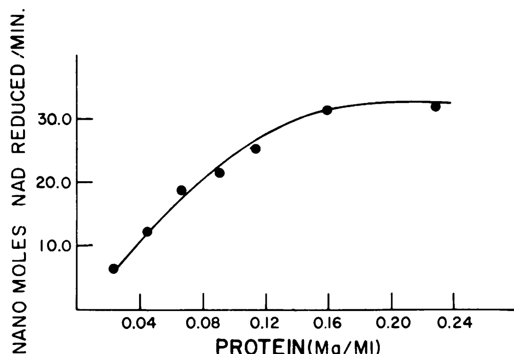

FIG. 4. *Glycerokinase activity of L_1 as a function of extract protein. Assay conditions are described in Materials and Methods.*

TABLE 3. *Effect of reaction mixture components on glycerokinase activity*

Incubation mixture	Initial activity (%)
Complete	100
Minus ATP-Mg	4
Minus extract	7
Minus L-α-GP dehydrogenase	0
Minus NAD	0
Minus hydrazine	94
Minus glycerol	10

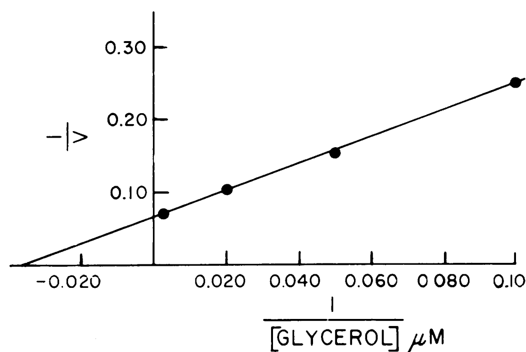

FIG. 5. *Double reciprocal plot of velocity versus substrate concentration for glycerokinase from strain L_1. Conditions of assay are presented in Methods section. Initial velocities are expressed in nanomoles per minute.*

ticulate fraction (106,000 × *g* pellet) of the cell extract, was independent of added pyridine nucleotides and was routinely measured by following the glycerophosphate-dependent reduction of the tetrazolium dye, MTT. These properties suggest that this enzyme is similar to the catabolic pyridine nucleotide-independent L-α-GP dehydrogenase previously described for *E. coli* by Lin et al. (14). As with the enzyme from *E. coli*, addition of PMS stimulated MTT reduction twofold (Table 4). Maximum stimulation (3.6-fold) occurred in the presence of both PMS and KCN. Approximately 90% of the observed activity was lost if either extract or GP was omitted from the assay. Activity was dependent upon the concentration of extract protein (0.01–0.12 mg of protein/ml) (Fig. 6) and, under the conditions of the assay, the enzyme exhibited a sp act of 200 nmol per min per mg of protein. An apparent K_m for GP of 2.3 mM was determined (Fig. 7). This value agrees with the K_m of 2.0 mM reported for the analogous enzyme from *E. coli* (8).

In contrast to strain L_1, the parental organism displayed significantly lower levels of both enzyme activities. A comparison of the values obtained from both organisms is given in Table 5.

DISCUSSION

The mutant (L_1) has been shown to assimilate glycerol during both photosynthetic and aerobic growth. Unlike the aerobic situation, anaerobic (light) glycerol assimilation requires added malate or CO_2 in the growth medium. These latter compounds presumably furnish

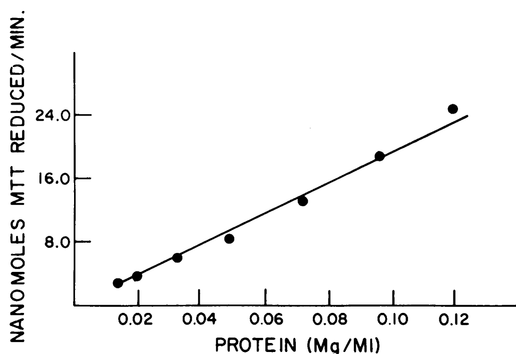

FIG. 6. *GP dehydrogenase activity of L_1 as a function of the extract protein concentration. Components of incubation mixture are given in Methods section.*

TABLE 4. *Effect of reaction mixture components on GP dehydrogenase activity*

Incubation mixture	Initial activity (%)
Complete	100
Minus MTT	0
Minus extract	9
Minus glycerophosphate	13
Minus PMS	40
Minus KCN	54
Minus PMS and KCN	27

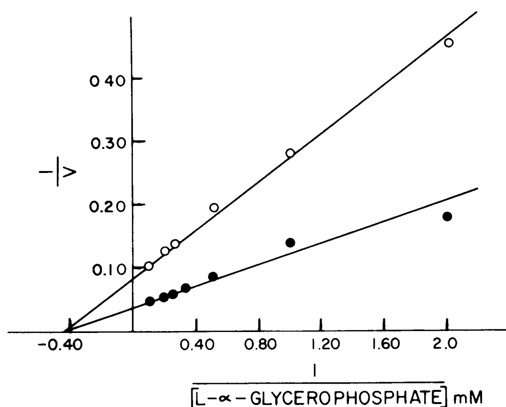

FIG. 7. *Double reciprocal plot of velocity versus substrate concentration for particulate GP dehydrogenase. Assay is described in Methods section. Symbols: 0.047 mg of protein/ml (○); 0.118 mg of protein/ml (●). Initial velocities are expressed in nanomoles per minute.*

TABLE 5. *Comparison of activities of the enzymes of the aerobic pathway of glycerol dissimilation*[a]

Strain	Glycerokinase activity[b]	GP dehydrogenase activity[b]
Wild Type	3.8	2.3
L_1	245.0	198.0

[a] Extracts were prepared from cells grown photosynthetically on malate-glycerol medium.
[b] Nanomoles per minute per milligram of protein.

the organism with an electron acceptor suitable for receiving reducing equivalents generated via anaerobic glycerol catabolism. Due to the presence of molecular oxygen or CO_2 as a terminal oxidant, this requirement is not present during aerobic growth on glycerol. This requirement for an oxidant during anaerobic glycerol dissimilation is not unique to the photosynthetic bacteria. *E. coli* has been shown to require nitrate (18) or fumarate (11) under similar conditions.

The ability of strain L_1 to assimilate glycerol is accompanied by markedly elevated levels of glycerokinase and pyridine nucleotide-independent GP dehydrogenase activities. The appearance of these enzymes suggests that this aerobic pathway is a dissimilatory route for glycerol in this organism. Furthermore, since the enzymes of this pathway are known to be inducible in other organisms (5), the elevated levels of these activities in L_1 may represent a secondary response to the original mutational event, the primary event being the elimination of a permeability barrier to glycerol. Finally, the possible selection of a regulatory mutant cannot be eliminated. It has been shown that mutants of *E. coli* which are lacking glycerokinase are unable to utilize glycerol for growth (7). Thus, it is feasible that strain L_1 possesses a regulatory mutation which has allowed the derepression of glycerokinase and the pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase. These possibilities are currently under investigation.

It is highly probable that the selection technique employed has resulted in the isolation of a multiple mutant, arising from a series of "fitter-type" mutations. Nitrosoguanidine mutagenesis failed to produce an organism with the glycerol-utilizing phenotype under either aerobic (dark) or anaerobic (light) selection conditions. However, it cannot be concluded from this work that other mutagenic agents would not produce an organism with the characteristics of strain L_1. In any event, a strain with the ability to metabolize glycerol should prove to be extremely useful in subsequent studies on lipid metabolism in this organism.

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