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Independent Constitutive Expression of the Aerobic and Anaerobic Pathways of Glycerol Catabolism in *Klebsiella aerogenes*

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Klebsiella aerogenes dissimilates glycerol aerobically via an inducible pathway initiated by an adenosine triphosphate-linked kinase that converts the substrate to *sn*-glycerol 3-phosphate. Phosphorylated glycerol is then dehydrogenated to dihydroxyacetone phosphate by an enzyme characteristic of a flavoprotein. Anaerobically, the organism dissimilates glycerol via an inducible pathway initiated by a nicotinamide adenine dinucleotide-linked dehydrogenase that converts the substrate to dihydroxyacetone. The keto product is then phosphorylated by another adenosine triphosphate-linked kinase. Two kinds of constitutive mutants have been isolated: one affecting the aerobic and the other the anaerobic pathway.

Glycerol is dissimilated by *Klebsiella aerogenes* 1033 by two separate pathways (Fig. 1), one serving primarily under aerobic and the other serving strictly under anaerobic conditions (5, 6). The aerobic system resembles the pathway described for *Escherichia coli* specified by the *glp* regulon (1-3). Through this route, glycerol is first phosphorylated by an adenosine triphosphate-dependent kinase. The product, *sn*-glycerol 3-phosphate (G3P), is then converted to dihydroxyacetone phosphate by a dehydrogenase characteristic of a flavoenzyme. Through the anaerobic route, glycerol is first dehydrogenated by a nicotinamide adenine dinucleotide-linked enzyme. The product, dihydroxyacetone, is then phosphorylated by an adenosine triphosphate-dependent kinase. The enzymes in both pathways are induced during growth on glycerol, but their relative cellular levels are highly dependent on the degree of aeration of growing cell cultures (5, 8). The purpose of this study is to find out whether each pathway can be independently derepressed genetically.

MATERIALS AND METHODS

Chemicals. The chemicals used in this study were high-purity reagent grade obtained from commercial sources. Suppliers of both the chemicals and growth supplements were the same as those previously published (7). Uniformly labeled [^{14}C]glycerol (112 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass., and [^{14}C]dihydroxyacetone (60 mCi/mmol) was from ICN Chemicals, Radioisotope Division, Irvine, Calif.

Bacteria. Strain 2103, an alkaline phosphatase-negative derivative of *K. aerogenes* 1033 (formerly known as *Aerobacter aerogenes* 1033) isolated and characterized in a previous report (7), served as the parental strain (wild type) in the present study. From this strain, two constitutive mutants were isolated after ethyl methane sulfonate treatment (4) of exponentially growing cells.

For the identification of clones with constitutive expression of the aerobic glycerol pathway, a mutagenized population was recycled in glucose minimal medium and spread (300 to 400 cells/plate) on minimal salts-tryptone (0.8 gm/liter) agar. After aerobic incubation for 48 h at 37 C, the plates were sprayed with an aqueous solution (pH 7.0) of chloramphenicol (0.5 mg/ml) and glycerol (0.5 g/ml). Sprayed plates were then incubated aerobically at 37 C for 1 h and sprayed again with a 1 M phosphate (pH 7.0) solution of triphenyltetrazolium chloride (10 mg/ml). Red colonies which appeared at a frequency of approximately one per 10^4 were picked, recloned, and subsequently grown in liquid culture for direct screening for the constitutive synthesis of enzymes in the aerobic glycerol pathway. Strain 2105 was thus isolated.

For the identification of clones with constitutive expression of the anaerobic glycerol pathway, a mutagenized population was recycled anaerobically on glucose minimal medium and spread (200 to 300 cells/plate) on minimal salts agar containing 0.5% casein hydrolysate and 5 mM sodium pyruvate. After anaerobic incubation (GasPak Systems, Becton-Dickinson and Co., Cockeysville, Md.) for 72 h at 37 C, the plates were sprayed with a 1 M phosphate (pH 7.0) solution containing chloramphenicol (0.5 mg/ml), glycerol (0.5 g/ml), and triphenyltetrazolium (10 mg/ml). Sprayed plates were again incubated anaerobically at 37 C for 2 to 3 h. Red colonies that appeared were picked, recloned, and grown in liquid culture for

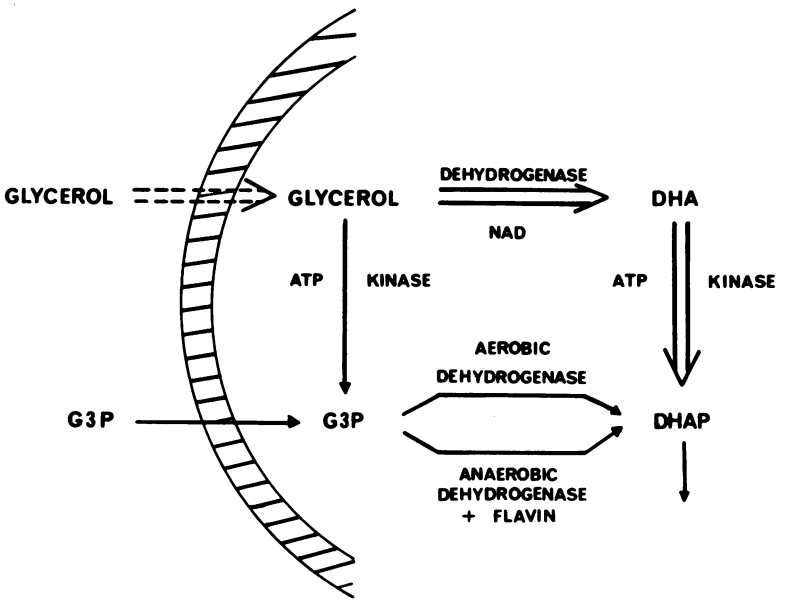

FIG. 1. *Pathways for glycerol and sn-glycerol 3-phosphate utilization in K. aerogenes 2103. The dissimilation of glycerol and G3P aerobically and that of G3P anaerobically are conducted via the pathway indicated by single-line arrows. The catabolism of glycerol anaerobically is depicted by double-lined arrows. Abbreviations: G3P, sn-glycerol 3-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; NAD, nicotinamide adenine dinucleotide; ATP, adenosine triphosphate.*

direct screening for the constitutive synthesis of enzymes in the anaerobic glycerol pathway. Strain 2106 was thus isolated.

Growth of cells and preparation of cell extracts. Simple carbon sources were added to a mineral medium (8) at the following concentrations: 10 mM glucose, 20 mM glycerol, 40 mM racemic α-glycerol phosphate (glycerol 3-phosphate and glycerol 1-phosphate) and 10 mM sodium pyruvate. When present in anaerobic cultures, sodium fumarate was added to a concentration of 20 mM as an exogenous electron acceptor. Casein acid hydrolysate (vitamin free) was added to a final concentration of 1.5% in aerobic cultures and 1.0% in anaerobic cultures for the growth of uninduced cell cultures. All cultures were grown in a 37 C warm room. Aerobic cultures were grown in Erlenmeyer flasks in volumes of 10 to 15% of the total capacity of the container. The flasks were shaken vigorously at approximately 240 cycles/min on rotary shakers. Anaerobic cultures were grown in flasks filled to capacity and stoppered to exclude air.

Extracts of aerobically and anaerobically grown cells harvested at densities of approximately 4×10^8 cells/ml were prepared by sonication and centrifugation (7). Dehydrogenase activities were measured spectrophotometrically, and kinase activities were measured by the detection of radioactive phosphorylated products (7).

RESULTS

Aerobic constitutive mutant. Strain 2105, detected as a glycerol-constitutive colony grown under aerobic conditions and designated tentatively as *glpR*$^-$, produced high levels of glycerol kinase and G3P dehydrogenase when grown aerobically in liquid culture with casein hydrolysate as carbon and energy source (Table 1). This constitutivity was also evident when the culture was grown anaerobically; however, the levels of these two enzymes appeared to be considerably reduced by growth in the absence of oxygen (Table 2). Substitution of the amino acid mixture with glycerol or G3P as the aerobic carbon and energy source resulted in further elevation of the levels of glycerol kinase and G3P dehydrogenase, indicating that the mutation did not completely abolish the functional repressor. The most striking observation in strain 2105, however, was that the regulatory mutation conferring constitutivity to *glp* pathway activities did not appreciably affect expression of the genes specifying the *dha* system that remained inducible as in the parental wild-type strain.

Anaerobic constitutive mutant. Strain 2106, detected as a constitutive colony grown under anaerobic conditions and tentatively designated as *dhaR*$^-$, exhibited essentially a reciprocal pattern in the expression of its genes involved in glycerol dissimilation. This strain produced high levels of glycerol dehydrogenase and dihydroxyacetone kinase when grown anaerobically

TABLE 1. *Specific enzyme activities in cells grown aerobically*

Strains	Genotype	Carbon source	Enzyme level[a]			
			Glycerol kinase	G3P dehydrogenase	Glycerol dehydrogenase	Dihydroxyacetone kinase
2103	Wild-type	Glucose	1.0 ± 0.8	2.0 ± 0.4	<1	0.3 ± 0.1
2105	*glpR*⁻		7.0 ± 0.7	74 ± 6.0	<1	0.2 ± 0.1
2106	*dhaR*⁻		0.2 ± 0.1	1.0 ± 0.1	3.0 ± 1.0	6.0 ± 0.7
2103	Wild-type	Casein	1.0 ± 0.1	2.0 ± 0.3	<1	0.3 ± 0.2
2105	*glpR*⁻	hydro-	240 ± 32	98 ± 0.3	<1	0.5 ± 0
2106	*dhaR*⁻	lysate	1.0 ± 0.5	2.0 ± 0.3	9.0 ± 7.0	6.0 ± 1.0
2103	Wild-type	Glycerol	380 ± 30	100 ± 6.0	<1	2.0 ± 0.7
2105	*glpR*⁻		530 ± 25	180 ± 0.8	<1	1.0 ± 0.3
2106	*dhaR*⁻		98 ± 16	37 ± 0.4	130 ± 14	63 ± 9.0
2103	Wild-type	G3P	530 ± 108	107 ± 3.0	<1	1.0 ± 0.5
2105	*glpR*⁻		630 ± 117	240 ± 21	<1	1.0 ± 0.5
2106	*dhaR*⁻		590 ± 75	120 ± 3.0	120 ± 23	17 ± 0.5

[a] All of the enzyme activities in this and the following tables are expressed as nanomoles/minute per milligram of protein.

TABLE 2. *Specific enzyme activities in cells grown anaerobically*

Strain	Genotype	Carbon source	Enzyme level			
			Glycerol kinase	G3P dehydrogenase	Glycerol dehydrogenase	Dihydroxyacetone kinase
2103	Wild-type	Glucose	0.1 ± 0.1	0.6 ± 0.1	12 ± 3.0	1.0 ± 0.2
2105	*glpR*⁻		0.2 ± 0.1	15 ± 1.0	8.0 ± 7.0	1.0 ± 0.3
2106	*dhaR*		0.1 ± 0.1	0.6 ± 0.2	110 ± 21	19 ± 4.0
2103	Wild-type	Casein	0.4 ± 0.1	5.0 ± 3.0	40 ± 3.0	2.0 ± 0.1
2105	*glpR*⁻	hydrolysate	13 ± 10	36 ± 2.0	32 ± 2.0	1.0 ± 0.1
2106	*dhaR*⁻	+ pyruvate	0.2 ± 0.1	2.0 ± 0.5	1,300 ± 150	120 ± 13
2103	Wild-type	Glycerol	3.0 ± 0.6	8.0 ± 5.0	1,300 ± 360	78 ± 7.0
2105	*glpR*⁻		9.0 ± 5.0	46 ± 6.0	1,300 ± 320	64 ± 12
2106	*dhaR*⁻		1.0 ± 0.6	6.0 ± 0.4	1,500 ± 250	87 ± 12
2103	Wild-type	G3P + fumarate	41 ± 2.8	120 ± 2.6	840 ± 17	6.4 ± 0.5
2105	*glpR*⁻		76 ± 1.4	100 ± 2.4	4,200 ± 860	13 ± 3.0
2106	*dhaR*⁻		15 ± 0.2	75 ± 0.1	4,600 ± 200	58 ± 1.6

in liquid culture with casein hydrolysate and sodium pyruvate as carbon and energy sources (Table 2).

This constitutivity was also evident when the culture was grown aerobically, although the levels of these two enzymes were considerably lower (Table 2). It should be mentioned that glycerol dehydrogenase is subject to specific inactivation in wild-type cells under conditions permitting aerobic generation of metabolic energy (5). This process probably also operates in strain 2106 and accounts for its low constitutive glycerol dehydrogenase levels under the aerobic conditions of growth.

Surprisingly, substitution of the amino acid mixture with G3P as carbon and energy source resulted in an almost threefold increase in the specific activity of glycerol dehydrogenase produced anaerobically by strain 2106 (Table 2). Glycerol itself under these conditions, however, produced only a minor increase in the level of the dehydrogenase. By contrast, both G3P and glycerol enhanced levels of the dehydrogenase and dihydroxyacetone kinase aerobically in

strain 2106, although the increases were relatively small.

As in the case of 2105, the effect of the constitutive mutation in strain 2106 was restricted to only one branch of the converging catabolic network.

Catabolite repression. Repressibility of both constitutive systems could still be demonstrated by growth on glucose either aerobically (Table 1) or anaerobically (Table 2), with the exception of dihydroxyacetone kinase in strain 2106 grown under aerobic conditions. The level of kinase in these cells was already markedly reduced by growth in the presence of oxygen (Tables 1 and 2). The addition of cyclic adenosine monophosphate (cAMP) to growth medium containing either glucose or casein hydrolysate as carbon and energy source resulted in only slight or modest increases in levels of most of the constitutively synthesized enzymes (Tables 3 and 4). The largest increases were observed for glycerol dehydrogenase in strain 2106, the level of which was increased 13-fold by cAMP during anaerobic growth on glucose, and for glycerol kinase in strain 2105, the level of which was increased threefold by cAMP additions during aerobic growth on glucose. Both of these enzymes were also the most sensitive to glucose repression (compare values of cells grown on casein hydrolysate versus those grown on glucose in Tables 1 and 2). It may be of physiological significance that the two enzymes initiating the metabolism of glycerol through each branch of the network were the most sensitive to catabolite repression.

DISCUSSION

In the present study, we have tentatively designated strain 2105 as *glpR*$^-$ on the assumption that the *glp* systems in *K. aerogenes* and *E.*

TABLE 3. *Specific enzyme activities in cells grown aerobically in the presence of 3.0 mM cAMP*[a]

Growth conditions and strains	Enzyme level			
	Glycerol kinase	G3P dehydrogenase	Glycerol dehydrogenase	Dihydroxyacetone kinase
Glucose				
2103	2.0 ± 0.8	2.0 ± 0.5	<1	0.2 ± 0.1
2105	27 ± 8.0	74 ± 9.0	<1	0.1 ± 0
2106	0.3 ± 0.1	1.0 ± 0.1	16 ± 4.0	16 ± 5.0
1.5 % Casein hydrolysate				
2103	0.2 ± 0.1	3.8 ± 1.0	<1	0.2 ± 0.1
2105	530 ± 5.0	110 ± 3.0	<1	0.5 ± 0.2
2106	0.1 ± 0	3.4 ± 1.0	13 ± 3.0	12 ± 3.0

[a] For values without added cAMP see Table 1.

TABLE 4. *Specific activities of glycerol catabolic enzymes grown anaerobically in the presence of 3.0 mM cAMP*[a]

Growth conditions and strains	Enzyme level			
	Glycerol kinase	G3P dehydrogenase	Glycerol dehydrogenase	Dihydroxyacetone kinase
Glucose				
2103	0.2 ± 0.1	0.8 ± 0.2	10 ± 2.0	1.0 ± 0.5
2105	0.6 ± 0.4	16 ± 1.0	17 ± 0	0.4 ± 0.1
2106	0.2 ± 0	2.0 ± 1.0	1300 ± 130	71 ± 1.0
1.0% Casein hyrolysate +pyruvate				
2103	0.7 ± 0.3	3.3 ± 1.0	28 ± 2.0	0.8 ± 0.5
2105	31 ± 1.0	37 ± 2.0	20 ± 1.0	0.9 ± 0.4
2106	0.2 ± 0.1	1.3 ± 1.0	2100 ± 110	140 ± 3.0

[a] For values without added cAMP see Table 2.

coli are homologous. The simultaneous derepression of glycerol kinase and G3P dehydrogenase, which belong to two separate operons in *E. coli*, together with parallel increases in G3P permease and flavin-stimulated G3P dehydrogenase activities (F. Ruch, unpublished data), provides good evidence that the mutation did indeed occur in the repressor gene. In contrast, the designation of strain 2106 as ***dhaR***$^-$ is only for temporary convenience, since it is not yet known whether or not glycerol dehydrogenase and dihydroxyacetone kinase are coded for by genes in separate operons and whether or not the expression of these genes is under negative control through a repressor protein. The disproportionate changes in the specific activities of glycerol dehydrogenase and dihydroxyacetone kinase resulting from variations of growth conditions does not permit the assignment of the two corresponding structural genes to different operons because of the existence of a specific inactivation process for glycerol dehydrogenase. Proper characterization of the nature of the regulation of the *dha* system will require fine-structure mapping by transduction and the availability of techniques permitting diploid analysis in this organism.

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