GENETIC STUDIES OF GLYCEROL CATABOLISM IN *PSEUDOMONAS AERUGINOSA*

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CHAPTER I

INTRODUCTION

The genus Pseudomonas has been known for many years to include species which are pathogens of plants, animals and man. The aerobic pseudomonads have also attracted the attention of microbial physiologists because of their range of habitats, the variety of biological types and their biochemical versatility. A very extensive study of the physiological characteristics of this group has recently been published (Stanier, et al., 1966). However, in spite of the extensive use of members of the genus Pseudomonas in studies of metabolism, little attention had been paid to the genetics of these organisms or to their metabolic control mechanisms until fairly recently.

Present knowledge of the genetic control of metabolism is based almost wholly upon studies with Escherichia coli. Jacob and Monod (1961) first proposed dual controls of enzyme synthesis in bacteria, in which the genetic material functions at two levels. The so-called structural genes determine the molecular structure of the proteins; regulator and operator genes control the rate of protein synthesis through the synthesis and action of specific repressors. The repressors are acted upon by specific metabolites and can be either inactivated (induction of enzyme synthesis) or activated (repression of enzyme synthesis). The synthesis of the enzymes of inducible catabolic pathways is initiated by the presence of substrate or structurally

similar inducer molecules which interact with the product of a regulatory gene, the repressor. In the absence of inducer, the repressor acts on the operator to prevent the functioning of the genes under its control which constitute an operon.

A third regulatory element was introduced into the operon model with the discovery of the promoter region (Jacob, et al., 1964), which is located outside (to the left) of the operator region and the structural genes which it controls and is believed to be contiguous to the operator (Ippen, et al., 1968; Miller, et al., 1968). Ippen, et al. (1968) proposed that the promoter determines the maximal level of expression for an operon. The promoter is the site of RNA polymerase binding and initiation of synthesis of messenger RNA. The promoter has also been shown to be the site which is sensitive to catabolite repression in the lac operon. Silverstone, et al. (1969) reported that a partial deletion of the promoter of this operon in E. coli made the operon insensitive to repression by glucose-6-phosphate, which decreases synthesis of β-galactosidase 20-fold in cells with an intact promoter.

A more recent development in the theory of metabolic controls involves the role of cyclic AMP (3', 5'-adenosine monophosphate). This molecule has been reported to influence a great variety of reactions in mammalian tissues and, more recently, has been implicated in control of synthesis of repressible enzymes in E. coli (Perlman and Pastan, 1968). The site of action of cyclic AMP was suggested to be the promoter region. Small deletions in this area could cause synthesis of β-galactosidase to be insensitive to stimulation by cyclic AMP (Pastan and Perlman, 1968). These data suggested that expression of catabolite-repressible enzyme synthesis is controlled by the internal level of

cyclic AMP. This compound is now known to be required for initiation of messenger RNA synthesis at the promoter site (de Crombrugghe, et al., 1971). The most detailed studies of genetic control mechanisms have been those of the lac operon in E. coli, and these findings have been summarized recently (Beckwith and Zipser, 1970). Metabolic controls of this and other pathways have been recently reviewed by Epstein and Beckwith (1968) and by Martin (1969).

While the detailed studies of genetic exchange mechanisms and of genetic control of enzyme synthesis in E. coli have advanced rapidly in recent years, much less progress has been made in applying these concepts to other organisms. Demerec (1964) pointed out the importance of extending such studies to other, unrelated microorganisms, especially with regard to determining the extent of gene clustering, because this type of genetic organization is fundamental to the operon concept as it was originally proposed. Holloway, et al. (1963) had reported that Pseudomonas shows a different pattern of gene distribution from that found in E. coli and Salmonella typhimurium. In both the latter organisms, the structural genes controlling sequential steps of many biosynthetic or catabolic pathways are frequently arranged contiguously on the genome. In Pseudomonas the early studies of Holloway and his associates indicated that, for the biosynthetic pathways, the related markers of any one pathway apparently lacked the close linkage typically found for functionally related genes in E. coli. Later, more detailed studies (Fargie and Holloway, 1965; Holloway, 1969) have shown that such related genes are often arranged in clusters consisting of only two or three genes, and in several cases genes within these clusters appear to be contiguous.

Marinus and Loutit (1969 a) reported that the structural genes for two enzymes of isoleucine-valine biosynthesis (acetohydroxy acid synthetase and reductoisomerase) are contiguous in Pseudomonas aeruginosa. A possible operator mutation (identified by its pleiotropic effects) mapped close to the cluster. The two enzymes were found to be subject to coordinate multivalent repression (Marinus and Loutit, 1969 b).

In Pseudomonas putida, the enzymes of tryptophan biosynthesis are coded by two unlinked clusters and a single gene unlinked to either cluster (Chakrabarty, et al., 1968; Gunsalus, et al., 1968). Crawford and Gunsalus (1966) found that a three-gene cluster includes the structural genes for anthranilate synthetase, phosphoribosyl transferase and indoleglycerolphosphate synthetase. These enzymes are noncoordinately repressed by tryptophan. Tryptophan synthetase A and B proteins are coded by a two-gene cluster and are inducible by the substrate indoleglycerolphosphate. A possible operator, mutation of which led to constitutive synthesis of the A and B proteins, was apparently linked closely to the two-gene cluster. The isolated gene codes for phosphoribosyl anthranilate isomerase. These and other genetic studies of Pseudomonas have been recently reviewed (Holloway, 1969; Holloway, et al., 1971).

The present study is concerned with the catabolic pathway for glycerol in P. aeruginosa. The major pathways for degradation of glycerol in bacteria are shown in Figure 1. In one reported pathway, glycerol is first phosphorylated to glycerophosphate, then oxidized to dihydroxyacetone phosphate (reactions 4 and 6). This pathway involves a glycerol kinase, and an L-α-glycerophosphate dehydrogenase

Abbreviations:

L-α-GlyP, L-α-Glycerophosphate

DHA, dihydroxyacetone

DHAP, dihydroxyacetone phosphate

GAP, glyceraldehyde-3-phosphate

Pyr, pyruvate

KDPG, 2-keto-3-deoxy-6-phosphogluconate

Enzymes:

1. Glycerol permease

2,4. Glycerol kinase

3,5. Glycerol dehydrogenase

6. L-α-Glycerophosphate dehydrogenase

7. Dihydroxyacetone kinase

8. Triose phosphate isomerase

9. Fructose diphosphate aldolase

10. Fructose diphosphatase

11. Phosphohexose isomerase

12. Glucose-6-phosphate dehydrogenase

13. 6-phosphogluconate dehydrase

14. KDPG aldolase

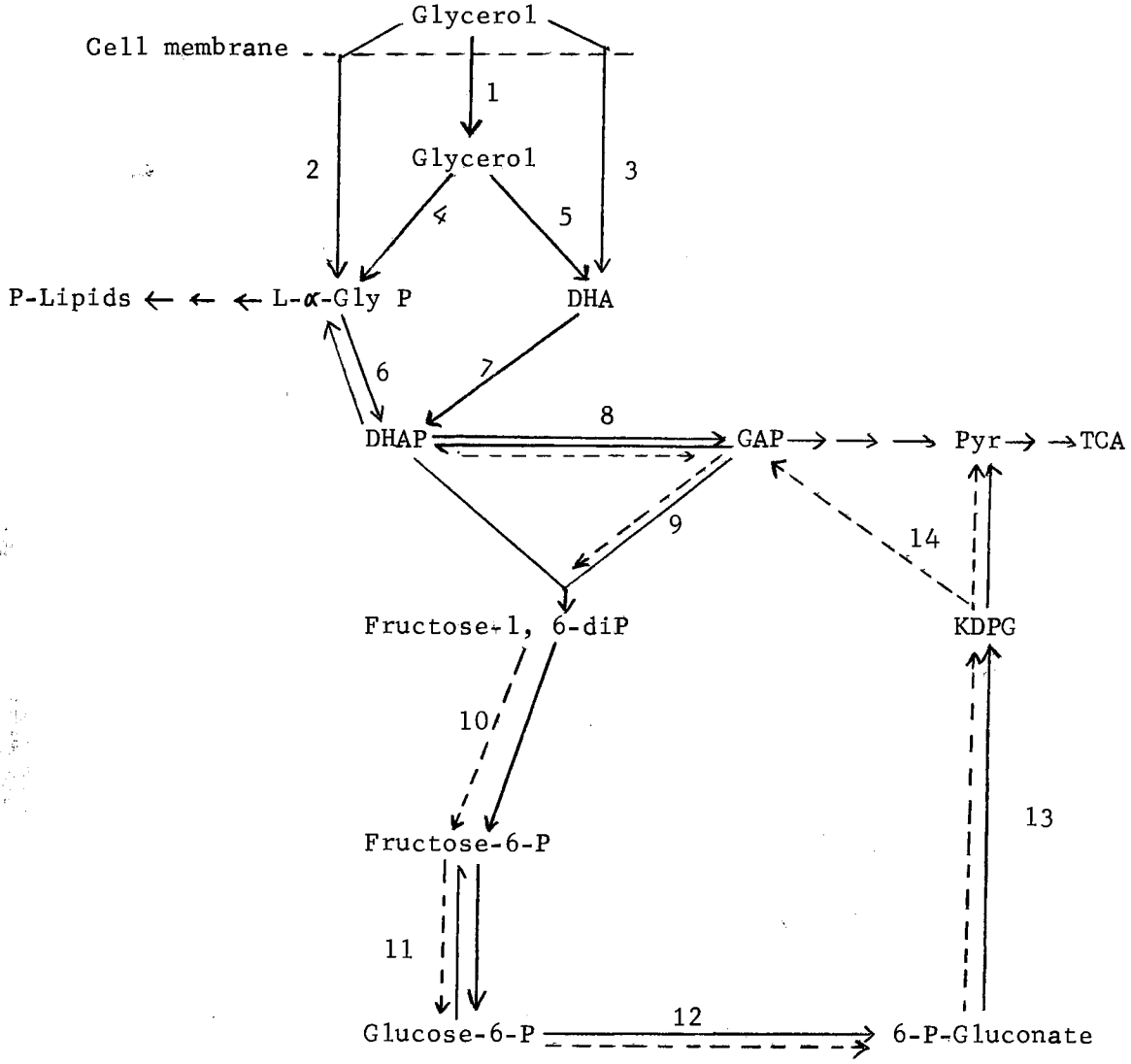


Figure 1. Major Pathways for Glycerol Metabolism in Bacteria.

(Magasanik, et al., 1953; Rush, et al., 1957). This pathway has been found in E. freundii (Mickelson and Shideman, 1947), Bacillus subtilis (Mindich, 1968), Streptococcus faecalis (Gunsalus and Umbreit, 1945; Jacobs and Van Demark, 1960), Mycobacterium smegmatis (Hunter, 1953), Aerobacter aerogenes (Burton and Kaplan, 1953); Magasanik et al., 1953), and Mycob. tuberculosis (Winder and Brennan, 1966). In another reported pathway, glycerol is oxidized to dihydroxyacetone, and is then phosphorylated to dihydroxyacetone phosphate (reactions 5 and 7). Enzymes in this pathway are an NAD-linked glycerol dehydrogenase and a dihydroxyacetone kinase. This pathway has been reported in E. coli (Asnis and Brodie, 1953), B. subtilis (Wiame, et al., 1954), S. faecalis (Gunsalus, 1947), Mycob. tuberculosis (Goldman, 1963), A. aerogenes (Burton and Kaplan, 1953), and Acetobacter suboxydans (Virtanen and Nordlund, 1933). The dihydroxyacetone phosphate formed by either reaction sequence is then converted to glyceraldehyde-3-phosphate by triose phosphate isomerase (reaction 8) and proceeds through the usual glycolytic pathway (the lower EMP pathway).

Both these reaction sequences may be found in the same organism, e.g., in A. aerogenes strain 1033 (Lin, et al., 1960), B. subtilis strains 168 and B24 (Mindich, 1968; Wiame, et al., 1954), and S. faecalis strain 10C1 (Jacobs and Van Demark, 1960). Kistler and Lin (1971) showed that E. coli is capable of dissimilating glycerol by either of two pathways depending upon conditions of growth; under aerobic conditions, a glycerol kinase and a particulate, pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase were utilized; under anaerobic conditions, a soluble, flavin-linked glycerophosphate dehydrogenase and the glycerol kinase were used.

The "anaerobic" pathway functioned only with nitrate or fumarate as electron acceptor. In this regard, E. coli differs from A. aerogenes which is capable of growing anaerobically on glycerol without an added electron acceptor, utilizing an NAD-linked glycerol dehydrogenase and a dihydroxyacetone kinase which are induced only under anaerobic conditions (Lin, et al., 1960).

Most of the biochemical and genetic studies of utilization of glycerol have been made using E. coli. Asnis and Brodie (1953) reported the conversion of glycerol to dihydroxyacetone by extracts of E. coli, but did not adequately describe the conditions of growth of the cells. Koch, et al. (1964) studied the aerobic catabolism of glycerol in E. coli strain K10. Glycerol kinase, L-α-glycerophosphate dehydrogenase, and an L-α-glycerophosphate transport system were shown to be responsible for the catabolism of glycerol and L-α-glycerophosphate. These three activities could be induced by growth on either glycerol or L-α-glycerophosphate.

Hayashi and Lin (1965) showed that a mutant of E. coli lacking glycerol kinase could not accumulate radioactivity from glycerol-^{14}C or grow on glycerol, and they also found that free diffusion of glycerol into cells was not rate-limiting for growth even at very low concentration. They concluded from these data that glycerol kinase is responsible for "capture" of glycerol. Sanno, et al. (1968) suggested that glycerol entered the cell by facilitated diffusion. They also showed that induction by glycerol or L-α-glycerophosphate was required for the entry of glycerol, but no induction was required for a constitutive mutant. These results were interpreted as evidence that the entry of glycerol into E. coli is not by simple diffusion, but is possibly

controlled by a specific gene product.

Genetic mapping of the glycerol loci in E. coli by Cozzarelli, et al. (1968) and by Kistler and Lin (1971) indicated that the structural genes for glycerol kinase and the aerobic L-α-glycerophosphate dehydrogenase and the locus controlling active transport of L-α-glycerophosphate are scattered on the genome. The postulated protein ("facilitator") required for facilitated diffusion of glycerol is specified by a locus closely linked to that for glycerol kinase. The structural gene for the soluble anaerobic L-α-glycerophosphate dehydrogenase is closely linked to the locus for transport of L-α-glycerophosphate. However, all these loci appear to be controlled by a single regulator gene which is closely linked to the aerobic L-α-glycerophosphate dehydrogenase locus and all are inducible by L-α-glycerophosphate (Kistler and Lin, 1971). Therefore, in E. coli, these genes are subject to a single control system but are not arranged contiguously as in the classic operon model. This type of physical arrangement of individual and/or clustered related genes under simultaneous, but not coordinate, control has been termed a regulon.

Studies of glycerol catabolism in P. aeruginosa were initiated in this laboratory by Cowen (1968). He showed that glycerol catabolism is inducible in this organism and found the enzymes glycerol kinase and L-α-glycerophosphate dehydrogenase in extracts of glycerol-grown cells. Unlike E. coli, P. aeruginosa was shown to be unable to transport L-α-glycerophosphate.

Tsay (1971) found that the enzymes for glycerol catabolism are not repressed by glucose in P. aeruginosa. Transport of glycerol was shown to require a binding protein inducible by glycerol and absent

in a transport-negative mutant (Tsay, et al., 1971).

Studies of the transport of glycerol were carried out by Brown (1972). Extensive study of both binding and transport of glycerol, as influenced by induction with a number of substrates and by inhibition with compounds of various types, led to the conclusion that P. aeruginosa has an active transport system for glycerol. A glycerol-specific binding protein was found to be inducible only by glycerol. A second binding protein induced by glycerol bound both glycerol and glucose, as well as pyruvate and lactate.

Another relationship between the catabolic pathways for glucose and glycerol in P. aeruginosa was suggested by the studies of Heath (1971). He studied the reactions involved in glucose metabolism in the wild type and in glucose-negative mutants. These reactions are also shown in Figure 1. He found that a large proportion of mutants isolated as glucose-negative were also unable to grow on glycerol and suggested that the reaction sequence shown in broken lines in the figure might be required for induction of the EMP enzymes necessary for growth on glycerol.

None of the previous studies of glycerol metabolism in P. aeruginosa have included transduction between glycerol mutants although, as pointed out previously, it is important for understanding of metabolic controls to determine whether related genes are clustered. Mutants used in previous studies had been either phage-resistant (Cowen, 1968) or too unstable for use in genetic studies (Tsay, 1971). Methods of mutant isolation developed by Heath (1971) and White (1972) allowed the isolation of a number of glycerol mutants which were sufficiently stable for use in transduction. It was the purpose of the present

investigation to determine the enzymatic lesions present in these glycerol-negative mutants and to carry out transductional analysis to test for linkage of the glycerol loci.

CHAPTER II

MATERIALS AND METHODS

Bacteria and Bacteriophage

Pseudomonas aeruginosa, strain 1 (designated hereafter as PA-1) was used in this study. The original culture of PA-1 was obtained from Dr. B. W. Holloway, Monash University, through Dr. Glenn Bulmer, Oklahoma Medical Research Center. Glycerol-negative mutants used in this study were derived from PA-1 by nitrosoguanidine treatment. Mutants Ag 10, Cg 1, Cg 2, GA 12, GA 22, GA 64, GA 73, AH 3, AH 8, AH 9, and AJ 4 were isolated by Dr. Floyd E. White, Jr.

The bacteriophage used for transduction studies was F 116 and was obtained from Dr. Holloway through Dr. Bulmer.

Media

Minimal medium was a modification of M-9 medium (Roberts, et al., 1957) and contained the following components (amounts per liter of solution): $Na_2HPO_4 \cdot 7H_2O$, 8.2 g; KH_2PO_4, 2.7 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; NH_4Cl, 1.0 g; $FeSO_4$, 0.1 per cent solution, 0.5 ml; distilled water to volume. The carbon sources were autoclaved or filter-sterilized separately as concentrated solutions and added to sterile minimal salts to give a final concentration of 0.5 per cent.

Nutrient broth (Difco) was prepared as suggested by the manufacturer. Pseudomonas phage broth (Holloway, et al., 1962) was used for

storage of phage suspensions and for making dilutions of phage stocks. It was composed of (amounts per liter): Difco nutrient broth, 8.0 g; Difco yeast extract, 5.0 g; NaCl, 5.0 g; distilled water to volume. For platings, the bottom layer contained 1.1 per cent Difco agar per liter of broth; plates contained 35 ml of agar. The top layer contained 0.65 per cent agar per liter of broth.

Lactate phage agar (Sutter, et al., 1963) was used for titration of phage suspensions and contained (amounts per liter); NaCl, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $NH_4H_2PO_4$, 1.0 g; K_2HPO_4, 1.0 g; sodium lactate, 60 per cent syrup, 2.0 ml; distilled water to volume. Bottom and top layer agars contained 1.1 per cent and 0.65 per cent agar, respectively. These were distributed in amounts of 35 ml per plate and 3.0 ml per tube, respectively.

Transduction medium (White, 1972) was composed of (amounts per liter): $Na_2HPO_4 \cdot 7H_2O$, 8.2 g; KH_2PO_4, 2.7 g; NH_4Cl, 1.0 g; $FeSO_4$, 0.1 per cent solution, 0.5 ml; NaCl, 5.0 g; Ionagar No. 2 (Colab), 9.0 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; distilled water to volume. The carbon source was added as a concentrated sterile solution to a final concentration of 0.5 per cent.

Growth of Bacteria

Stock cultures were maintained on nutrient agar slants by periodic transfer; the cultures were stored at $4^{\circ}C$ after growth. All cultures were incubated at $37^{\circ}C$ and liquid cultures were incubated on a reciprocal shaker. Growth of liquid cultures was measured as optical density at 540 nm against an appropriate blank with a Coleman Junior Spectrophotometer, Model D; an optical density of 0.220 (18 mm diameter

tube) was equivalent to 10^9 cells per ml.

Minimal salts medium (i.e., without added carbon source) was used for diluting and washing bacterial suspensions.

Preparation and Titration of Phage Plate Stocks

Phage plate stocks were prepared by a modification of the procedure of Swanstrom and Adams (1951). One drop of log phase cells grown in Difco nutrient broth and 0.1 ml of a phage suspension containing 5×10^6 plaque-forming units (PFU) per ml were added to 3.0 ml of Pseudomonas phage agar (top layer), mixed, and poured over 35 ml of bottom-layer Pseudomonas phage agar. After 12 to 18 hours incubation at 37°C, the phage were soaked off by adding 5.0 ml of phage broth to each plate. After 30 minutes, the broth was pipetted from the plates and cells and debris were removed by centrifugation. The supernatant fluid was filtered through a Millipore filter (HA, 0.45 μm pore size). This procedure yielded phage plate stocks with titers of approximately 10^{10} to 10^{11} PFU per ml. Phage plate stocks containing less than 10^{10} PFU per ml were not used for transduction.

For determining the titer of phage suspensions, a 0.1 ml volume of appropriately diluted phage suspension was added to 3.0 ml of top layer lactate phage agar along with one drop of a log-phase culture of PA-1. This was mixed and poured over the bottom layer. After the agar had solidified, plates were inverted and incubated at 37°C. Plaques were counted after approximately 12 hours.

Isolation of Glycerol Mutants

The procedure used for mutagenesis was a modification of the method

of Adelberg, et al. (1965). PA-1 was inoculated into 6 ml of nutrient broth and grown to approximately 10^9 cells per ml. The cells were centrifuged in the cold at 8000 rpm for 15 minutes, washed with 5 ml of 0.05 M, pH 6.0, sodium acetate buffer, resuspended in 6 ml of sodium acetate buffer which contained nitrosoguanidine at 2 mg/per ml, and incubated 30 minutes on a shaker at 37°C. The cells were collected by centrifugation in the cold (4°C), washed with minimal salts (without carbon source or $MgCl_2$) and resuspended in nutrient broth; they were then incubated at 37°C on a shaker. After 3 hours, cells were collected by centrifugation, washed with minimal salts and resuspended in glycerol minimal medium. Three ml of this cell suspension were added to 3 ml of antibiotic-containing glycerol minimal medium. The final concentrations were: D-cycloserine, 500 μg per ml, and carbenicillin, 100 μg per ml. The cells were incubated for 14 to 18 hours. A series of dilutions was plated in triplicate on glucose minimal agar.

After colonies appeared on glucose minimal medium, they were picked individually from plates with 100 to 200 colonies with a sterile needle and transferred to plates containing glycerol minimal agar, lactate minimal agar, and glucose minimal agar. This was done by placing a graph paper template below the plates in order to provide an orderly array of point inoculations. Colonies which grew normally on the glucose medium, but only slightly or not at all on glycerol, were selected as glycerol-negative mutants.

Selected mutants were tested again in glycerol minimal liquid medium and on glycerol minimal agar medium. Cultures of the confirmed mutants were used to prepare phage stocks for transduction and cell-free extracts for enzyme assays.

Chemicals

The following chemicals were purchased from Calbiochem: Tris (hydroxymethyl) aminomethane (Tris), adenosine 5'-triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), uniformly labeled ^{14}C-glycerol and the diethyl acetal barium salt of D,L-glyceraldehyde-3-phosphate which was converted to the free acid by the manufacturer's suggested procedure.

Carbenicillin was a gift from Beecham Pharmaceuticals. D-cycloserine was obtained from Mann Research Laboratories. N-methyl-N'-nitro-N-nitrosoguanidine was the product of Aldrich Chemical Company. Phenazine methosulfate and 3 (4,5 dimethyl thiazolyl 1-2) 2,5 diphenyl tetrazolium bromide (MTT) were products of Nutritional Biochemicals Corporation. Nicotinamide adenine dinucleotide phosphate (NADP) was obtained from Sigma Chemical Company. D-Glyceraldehyde was obtained from Schwartz Mann.

Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) and L-α-glycerophosphate dehydrogenase (rabbit muscle) were obtained from Sigma Chemical Company.

Protein Determination

The protein content of cell-free extracts was determined by the method of Sutherland et al. (1949). The extracts were diluted 1:20 and aliquots were made up to 1.0 ml with distilled water. Five ml of freshly prepared reagent containing 1.0 ml of 4 per cent sodium potassium tartrate, 1.0 ml of 2 per cent $CuSO_4 \cdot 5H_2O$, and 100 ml of 4 per cent Na_2CO_3 were added. After 40 minutes incubation at room temperature, 0.5 ml of a two-fold dilution of 2 N phenol reagent

(Fisher Scientific Company) in water was added and the tubes were mixed immediately. After at least 15 minutes at room temperature, the optical density was determined at 660 nm. Standards contained 30, 60, and 90 μg per ml bovine serum albumin. A water blank and a set of standards were included in each assay.

Gregory and Sajdera (1970) reported that certain buffers can affect color formation from protein; therefore, the protein standard solution was prepared in the 0.02 M Tris buffer, pH 8.5, which was used to make cell-free extracts.

Preparation of Cell-free Extracts

Cells were grown in 200 to 300 ml of the minimal medium containing 0.5 per cent glucose and 0.5 per cent glycerol, harvested by centrifugation, and washed twice with 0.85 per cent NaCl at 4°C. The final pellets were frozen at -20°C for no more than one week.

Frozen cell pellets were thawed in 5 to 10 ml of 0.02 M Tris buffer, pH 8.5. The cells were broken with a Bronson sonifier by exposure to 15-second bursts of sonic energy with intermittent cooling in an ice bath.

Whole cells and cell debris were removed by centrifugation at 4°C. These crude extracts were used directly for enzyme assays.

Enzyme Assays

All enzyme assays were performed spectrophotometrically with a Cary Model 14 dual beam recording spectrophotometer (Applied Physics Corporation). For each assay, the blank and sample cuvettes differed only by the substitution of water for substrate in the blank. The

glycerol dehydrogenase assay was carried out in microcuvettes, using a total volume of 1.0 ml. Total volume for all other assays was 3.0 ml. A unit of enzyme was defined as that amount required to convert one nanomole of substrate to product per minute under the specified conditions.

Assays developed during this investigation for enzymes not previously reported for bacterial metabolism of glycerol will be described in the following chapter.

Glycerol Dehydrogenase

This assay was based on that described by Goldman (1963) for a different glycerol dehydrogenase, but was modified for use in the present study. The sample tube contained: 0.5 to 0.7 ml of 0.1 M Tris buffer, pH 9.1; 0.1 ml of 0.02 M NADP; 0.3 ml of cell-free extract; and 0.1 ml of 0.01 M glycerol. The millimolar extinction coefficient used for calculation of specific activity was 6.2 (Dawson et al., 1959).

Glycerol Kinase

A modification of the method of Lin, et al. (1962) was used to measure the phosphorylation of glycerol by coupling with added L-α-glycerophosphate dehydrogenase. The substrate was omitted in the blank. The sample tube contained: 1.2 ml of 0.1 M Tris buffer, pH 7.5; 0.1 ml of 0.05 M ATP; 0.2 ml of 0.02 M NAD; 0.1 ml (approximately 5 units) of L-α-glycerophosphate dehydrogenase (rabbit muscle); 0.3 ml of 3 N hydrazine; 0.6 ml of 0.1 M $MgCl_2$; 0.2 ml of cell-free extract; and 0.3 ml of 0.1 M glycerol. Formation of NADH was followed at 340 nm. The millimolar extinction coefficient used for calculation of specific activity was 6.2 (Dawson, et al., 1959).

L-α-glycerophosphate Dehydrogenase

This assay was also a modification of that of Lin, et al. (1962). The sample tube contained: 2.4 ml of 0.1 M Tris buffer, pH 7.5; 0.2 ml of 0.15 M KCN; 0.2 ml of cell-free extract; 0.1 ml of a 1 mg per ml solution of MTT; and 0.1 ml of 1.0 M DL-α-glycerophosphate. The enzyme activity was assayed by measuring the rate of reduction of the tetrazolium dye, MTT (thiazolyl blue), to its formazan which absorbed maximally at 550 nm. Measurement of dehydrogenase activity was enhanced by the addition of KCN which prevented the passage of electrons through the cytochrome system. The millimolar extinction coefficient used for calculation of specific activity was 9.9 (Sowerby and Ottaway, 1966).

Transduction

The transduction technique of Murphy and Rosenblum (1964) was used with modification. Thirteen mutants were used and each mutant was crossed with the wild type and with all other mutant strains. Each mutant served as donor and recipient, respectively. Recipient cells were washed from fresh nutrient agar slants with 2 ml of minimal medium (without carbon source) and 0.1 ml aliquots were spread on pre-dried glycerol minimal medium plates. One drop of each donor phage stock was spotted at a marked location on the surface of each plate. Phage suspensions were also spotted on nutrient agar plates without cells to test the sterility of the phage plate stock. The plates were incubated at room temperature and transductants were scored after approximately three to seven days incubation.

Transport of Glycerol-U-^{14}C

The method used for measurement of transport of glycerol-U-^{14}C was developed by Brown (1972). The desired strain of P. aeruginosa was grown in minimal medium containing 0.5 per cent glucose and 0.5 per cent glycerol. The cells were centrifuged, and washed with 0.85 per cent saline two times and resuspended in minimal medium (without carbon source) to a per cent transmittance of 45. A 0.1 ml volume of a 10^{-6} dilution of this cell suspension was spread onto each of three glucose minimal medium plates, and after 24 hours of incubation at 37°C the colonies were counted and the average count used to calculate concentration of viable cells. A 12.45 ml volume of the cell suspension was placed in a 125 ml Erlenmeyer flask. The flask was then incubated for 10 minutes at 37°C and 134 oscillations per minute in a shaking water bath.

At zero time, 0.05 ml of solution containing 7.95 nmoles of glycerol-U-^{14}C was added to the 12.45 ml of cell suspension. This resulted in a concentration of approximately 0.6 nmoles/ml of glycerol-U-^{14}C. At 30, 60 and 120 seconds after addition of glycerol, 1.0 ml samples were removed and filtered immediately through a pre-wetted Gelman Metricel filter (0.45 μm pore size) placed on a metal screen in a Millipore filter funnel apparatus. The vacuum was opened to each filter tower 25 seconds before sampling and filters were washed with 1.0 ml of minimal medium twice at 60 seconds and 105 seconds after sampling. After all washings had been completed, filters were removed, placed in scintillation vials and dried with hot air from a blower. The filters were then crushed and 10 ml of scintillation fluid were added to each vial. As a standard, 0.01 ml of the

glycerol-U-^{14}C solution was placed in a separate vial in 10 ml of scintillation fluid. Thirty minutes after addition of scintillation fluid, the vials were shaken vigorously to emulsify the filter particles. The vials were then allowed to stand at least four hours at room temperature without being disturbed before being counted on a Nuclear Chicago Model 720 counter.

The scintillation fluid used was a 1:1 mixture of Aquasol (New England Nuclear) and a solution of 4 g/l of 2,3-diphenoloxazole (PPO) and 200 mg/l of 1,4-bis [2-(5-phenyloxazolyl) benzene] (POPOP) in a 4:6 (v:v) mixture of absolute ethanol and toluene.

Since all experiments were performed under identical standardized conditions using approximately equal numbers of cells, transport data are reported as cpm per 10^9 cells. The 1.0 ml sample used for filtration contained approximately 1.5 x 10^9 cells.

CHAPTER III

EXPERIMENTAL RESULTS

Isolation of Glycerol-negative Mutants

Nitrosoguanidine-treated PA-1 cells were enriched, as described previously, for 12-18 hours in glycerol minimal medium containing D-cycloserine and carbenicillin at final concentrations of 500 and 100 μg per ml, respectively. The advantage of using a combination of antibiotics was that growing cells resistant to one antibiotic could be killed by the other; double mutants resistant to both antibiotics should be very rare.

Eleven glycerol-negative mutants were isolated by this procedure. Selected mutants were tested again in glycerol and glucose liquid minimal medium, respectively, for confirmation. It was found that all except one of the glycerol-negative mutants grew in 18-24 hours in both glycerol and glucose media; i.e. all the mutants reverted except mutant 8. Twelve other glycerol-negative mutants, isolated by Floyd E. White, Jr., were used in the present study along with mutant 8, isolated by the procedures described above.

Characterization of Glycerol-negative Mutants

Growth Studies

Each mutant was tested for its ability to grow on minimal medium

with four different carbon sources. The cells were grown on nutrient agar slants and washed off with 2 ml of minimal salts medium. One drop of this cell suspension was added to 6 ml of glucose, lactate, succinate and glycerol liquid minimal media, respectively. Growth was measured as optical density at 540 nm in 18 mm-diameter tubes. Readings were made at intervals of 4 hours during aeration and a final reading was made at 24 hours. The 24 hr readings are given in Table I.

All except two of the mutants grew well in glucose, succinate and lactate media, and grew only slightly or not at all in glycerol minimal medium. Mutants Cg 1 and Cg 2 grew in neither glucose nor glycerol minimal medium, but grew well in succinate and lactate minimal media or in minimal medium with 0.5% yeast extract added. These mutants were therefore grown in medium containing yeast extract in addition to the usual carbon sources when cells were prepared for enzyme assay.

Enzyme Activities

It was necessary to examine the cells used for preparation of cell-free extracts to determine whether reversion to wild-type had occurred during growth of the culture. Cells were grown as described previously to an optical density of approximately 0.22. Before harvesting the cells by centrifugation, one drop of the culture was inoculated into glycerol minimal medium and placed on the shaker at 37°C. The remainder of the culture was then centrifuged, washed and stored at -20°C as a pellet. If the cells inoculated into glycerol minimal medium did not grow within 24 hr, the pellet was then used to prepare a cell-free extract for enzyme assays.

TABLE I

GROWTH OF WILD TYPE AND MUTANTS ON DIFFERENT CARBON SOURCES

Organism	Growth Media			
	Glycerol	Glucose	Succinate	Lactate
PA-1	1.048	1.046	0.638	1.097
Ag 10	0.041	1.097	0.658	1.155
Cg 1	0.076	0.036	0.569	0.824
Cg 2	0.081	0.022	0.638	0.959
8	0.181	0.921	0.658	0.959
GA 3	0.056	1.000	0.638	0.921
GA 12	0.046	1.000	0.638	1.222
GA 22	0.222	1.046	0.638	0.886
GA 64	0.046	1.046	0.556	0.921
GA 73	0.027	0.824	0.699	1.155
AH 3	0.051	0.959	0.638	1.097
AH 8	0.032	0.850	0.602	1.222
AH 9	0.097	0.886	0.699	1.097
AJ 4	0.056	1.000	0.620	1.046

OD was read at 540 nm after 24 hr incubation.
Carbon sources were used at a concentration of 0.5 per cent in minimal medium.

Enzyme activities were measured on the Cary recording spectrophotometer at appropriate wavelengths. The enzymes glycerol dehydrogenase, L-α-glycerophosphate dehydrogenase and glycerol kinase had been detected previously in glycerol-grown PA-1. Glyceraldehyde dehydrogenase activity was first detected in the present study, and the assay procedure for this enzyme will be described in a later section. The enzyme activities of the mutants were measured and compared to levels found in extracts of PA-1 prepared concurrently. Data for all specific enzyme activities are shown in Table II, and in Table III these activities are shown as percentages of the activity found in extract from wild type cells. For assays which were repeated, the specific activity shown is the highest detected.

In a catabolic pathway in which enzyme activities are inducible, it is somewhat difficult to determine the significance of variations in enzyme levels compared with those of the wild type which can grow on the substrate and may be more completely induced. However, it seems reasonable to assume that enzyme activities less than 20 percent of the wild type level are due to mutation rather than to inefficient induction. Using this arbitrary limit as a criterion, the enzymatic defects in the mutants may be identified as follows:

GA 73 lacks glycerol kinase.

AJ 4 and AH 3 lack L-α-glycerophosphate dehydrogenase.

GA 22 lacks glycerol dehydrogenase.

AH 8 lacks both glycerol dehydrogenase, and L-α-glycerophosphate dehydrogenase.

AH 9 and GA 12 lack both glycerol kinase and L-α-glycerophosphate dehydrogenase and have relatively low glycerol dehydrogenase activity.

TABLE II

SPECIFIC ACTIVITIES OF GLYCEROL ENZYMES IN CELL-FREE EXTRACTS OF WILD TYPE AND MUTANTS

Organism	Enzyme			
	GK	GPDH	GDH	GADH
PA-1	11.7	24.2	1.30	13.4
GA 73	1.8	6.7	2.15	15.3
AJ 4	8.4	2.2	1.12	13.7
AH 3	8.8	4.2	0.50	17.2
GA 22	6.5	8.7	0	12.6
AH 8	9.2	0.65	0	13.4
8	3.1	8.6	0.62	16.0
AH 9	1.3	3.2	0.48	19.0
GA 12	0.4	0.60	0.36	11.4
Ag 10	0	0	0.19	15.9
GA 3	0	0	0	13.5
GA 64	0	0	0	17.2
Cg 1	4.5	11.9	0.41	15.8
Cg 2	9.9	9.7	0.40	12.6

Abbreviations: GK, glycerol kinase; GPDH, L-α-glycerophosphate dehydrogenase; GDH, glycerol dehydrogenase; GADH, glyceraldehyde dehydrogenase.

Specific activities are nmole/min/mg protein. A specific activity of 0 indicates that no activity was detected.

TABLE III

ENZYME ACTIVITIES OF MUTANTS CALCULATED AS PERCENT OF WILD TYPE ACTIVITY

Organism	Enzyme			
	GK	GPDH	GDH	GADH
GA 73	16	28	165	114
AJ 4	72	9	86	102
AH 3	75	17	38	128
GA 22	56	36	0	94
AH 8	79	3	0	100
8	27	36	48	119
AH 9	11	13	37	142
GA 12	3	2	28	85
Ag 10	0	0	14	119
GA 3	0	0	0	100
GA 64	0	0	0	128
Cg 1	38	49	32	118
Cg 2	85	40	31	93

Abbreviations: GK, glycerol kinase; GPDH, L-α-glycerophosphate dehydrogenase; GDH, glycerol dehydrogenase; GADH, glyceraldehyde dehydrogenase.

Activities which are less than 20 per cent of the wild type level are underlined.

Ag 10, GA 3 and GA 64 lack glycerol kinase, L-α-glycerophosphate dehydrogenase and glycerol dehydrogenase.

Mutants Cg 1, Cg 2, and 8 have some activity for all the enzymes mentioned above, but these activities are all relatively low with the exception of the glycerol kinase of Cg 2. None of the mutants is defective in glyceraldehyde dehydrogenase activity.

Transport of Glycerol

All mutants not previously used in transport studies by Brown (1972) were tested for the ability to transport glycerol in uptake studies using glycerol-U-^{14}C. Each mutant was grown in 25 ml of minimal medium containing 0.5 percent each of glycerol and glucose (0.5 percent yeast extract was added for mutants Cg 1 and Cg 2). The cultures were aerated in 250 ml Erlenmeyer flasks fitted with test tube side arms so that growth could be followed by reading optical density. When the transmittance reached approximately 45 percent, the cells were harvested and used in uptake experiments as described in Chapter II.

The data for these studies are given in Table IV. Mutants GA 73 and GA 22 had been previously determined by Brown to have transport ability equivalent to that of PA-1, while AH 8 had been shown to have lowered transport ability. This was correlated with the absence of the specific glycerol binding protein. Of the mutants used in the present study of transport, only Ag 10 appeared to be totally inactive in the transport of glycerol. In this respect, it is similar to mutant 623 (Tsay, et al., 1971). Mutants AH 3, GA 12 and GA 64 had very low transport activity and the transport activity of mutant AH 9

TABLE IV

TRANSPORT OF GLYCEROL-U-^{14}C BY
GLYCEROL-NEGATIVE MUTANTS

Organism	Counts/min/10^9 Cells
AJ 4	3720
AH 3	870
8	3740
AH 9	1770
GA 12	1060
Ag 10	80
GA 3	4170
GA 64	760
Cg 1	5872
Cg 2	5183

Uptake level in wild type cells under the same conditions is approximately 4000 counts/min/10^9 cells.

was also quite low. All other mutants, GA 3, AJ 4, Cg 1, Cg 2 and 8 had transport activity at the wild type level (approximately 4000 cpm/10^9 cells). Only the one-minute values are given in the table since these appeared to be representative of the transport activity. Each sample was counted five times and the counts were averaged and corrected for background.

Alternate Pathway of Glycerol Metabolism

Studies by Cowen (1968) and Tsay (1971) had shown that the catabolism of glycerol in P. aeruginosa proceeds through glycerol kinase, L-α-glycerophosphate and triose phosphate isomerase. Brown's finding that a mutant lacking glycerol kinase produced large amounts of $^{14}CO_2$ from ^{14}C-glycerol indicated that P. aeruginosa must have an additional route for metabolism of glycerol. Assays for possible enzymes utilizing glycerol as substrate resulted in the finding that freshly prepared extracts of PA-1 contain an extremely labile NADP-linked glycerol dehydrogenase (Brown, 1972). The product has been identified as glyceraldehyde (Brown, personal communication). Accordingly, Brown suggested that the series of reactions shown in Figure 2 could account for the release of $^{14}CO_2$ from ^{14}C-glycerol by a kinase-less mutant.

It was important in the present investigation to determine the fate of the glyceraldehyde formed by the glycerol dehydrogenase reaction. Fresh extracts of PA-1 were tested for the presence of a dehydrogenase and a kinase for glyceraldehyde. The assay system for detection of dehydrogenase activity contained: cell-free extract, Tris buffer, pH 7.5, D-glyceraldehyde as substrate and an electron acceptor.

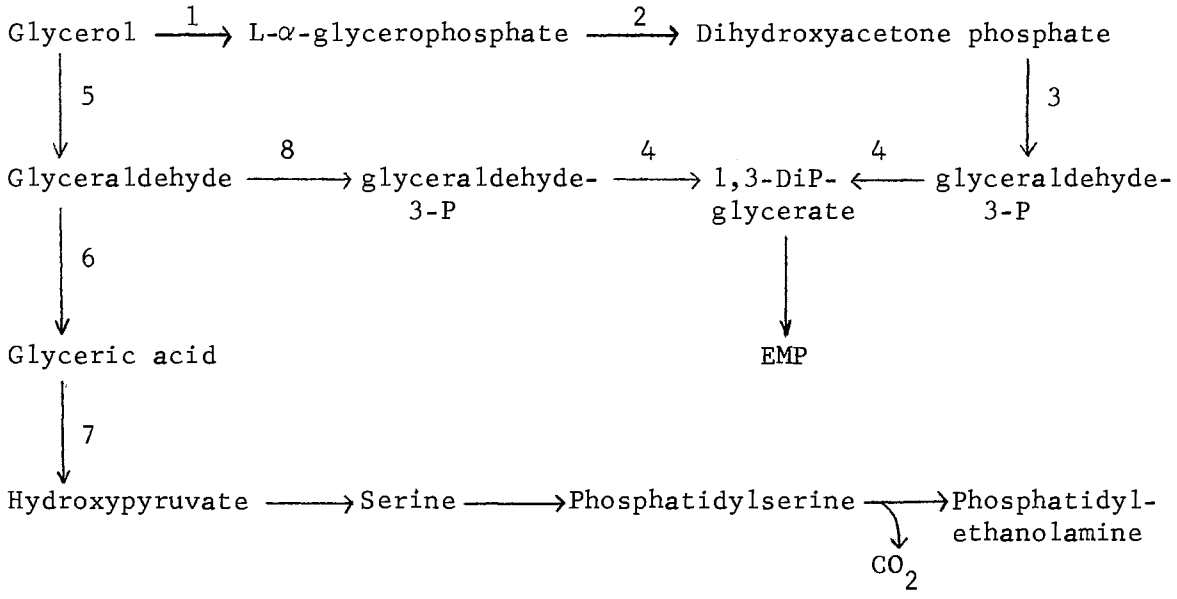


Figure 2. Possible Alternate Routes for Glycerol Metabolism in Pseudomonas aeruginosa.

Enzymes:

1. Glycerol kinase
2. L-α-glycerophosphate dehydrogenase
3. Triose phosphate isomerase
4. Glyceraldehyde-3-P dehydrogenase
5. Glycerol dehydrogenase
6. Glyceraldehyde dehydrogenase
7. Glycerate dehydrogenase
8. Glyceraldehyde kinase

No activity was detected when either NAD or NADP was added to the assay system and optical density at 340 nm was recorded. However, the tetrazolium dye, MTT, was reduced rapidly. The rate of reduction was increased by addition of phenazine methosulfate as an intermediary election carrier and was further increased by the addition of cyanide to prevent passage of electrons through the cytochrome system. Therefore, it was concluded that P. aeruginosa possesses an active D-glyceraldehyde dehydrogenase which is not linked to either NAD or NADP. This dehydrogenase was also found to be somewhat labile since no activity could be detected in an extract which had been stored at $-20^{\circ}C$ for approximately six weeks.

The assay system used for measurement of the D-glyceraldehyde dehydrogenase activity shown in Table II contained 2.1 ml of 0.1 M Tris buffer, pH 7.5; 0.2 ml of 0.15 M KCN; 0.2 ml of cell-free extract; 0.2 ml of 1 mg/ml MTT; 0.1 ml of 3 mg/ml phenazine methosulfate; 0.3 ml of 0.1 M D-glyceraldehyde. Substrate was replaced by buffer in the blank. Fresh extracts were prepared for assay of this enzyme.

The other possible route for further metabolism of D-glyceraldehyde would involve the enzyme D-glyceraldehyde kinase which would form glyceraldehyde-3-phosphate. The assay system used for this enzyme was similar to that employed for glycerol kinase except that the enzyme added for coupling the kinase to NAD reduction for spectrophotometric assay was glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), and the components added were those used by Heath (1971) for assay of phosphofructokinase coupled to the same dehydrogenase. The assay system included: 1.4 ml of 0.1 M Tris buffer, pH 7.5; 0.1 ml of 0.05 M ATP; 0.2 ml of cell-free extract; 0.2 ml of 0.02 M NAD; 0.6 ml of 0.1 M $MgCl_2$;

0.1 ml of 0.5 M Na_2HASO_4; 0.1 ml of a solution of glyceraldehyde-3-phosphate dehydrogenase containing approximately 100 EU per ml; 0.3 ml of 0.1 M D-glyceraldehyde. Fresh extract was prepared for use in the assay. No activity was detected. It has been found with other assays of kinases coupled to dehydrogenase (Heath, 1971; Brown, 1972) that preincubation of extract, buffer and ATP greatly enhances the activity measured. Therefore, preincubation for 12-15 min was included in the procedure; again no activity was detected. As a control to show that production of glyceraldehyde-3-phosphate would have been detected had it occurred, 0.1 ml of 0.05 M glyceraldehyde-3-phosphate was added to the sample cuvette after approximately 20 min. incubation (15 min. preincubation of extract, buffer and ATP plus 5 min. incubation after addition of the remaining components). Rapid reduction of NAD occurred immediately. Therefore, it was concluded that P. aeruginosa does not possess D-glyceraldehyde kinase activity.

Transduction Studies

The spot plate method of Murphy and Rosenblum (1964) was used in transduction studies as described previously. Each mutant served as recipient and donor, respectively, and was crossed with the wild type and with all other mutants. The results were recorded as the number of colonies appearing within the area of the phage spot and are presented in Table V. Each number is the highest observed in repeated experiments. These numbers of colonies do not represent recombination frequencies, but rather numbers of transductants. The wild type PA-1 as donor crossed with most mutants producing large numbers of transductants. Four mutants, AH 3, GA 12, GA 3 and GA 64

TABLE V

TRANSDUCTION BETWEEN GLYCEROL MUTANTS

Donor	Recipient												
	GA 73	AJ 4	AH 3	GA 22	AH 8	8	AH 9	GA 12	Ag 10	GA 3	GA 64	Cg 1	Cg 2
GA 73	0	30	0	0	10	0	0	19	11	0	0	6	6
AJ 4	132	0	0	0	97	0	0	8	10	0	0	0	2
AH 3	TNTC	4	0	0	101	0	0	97	0	0	0	20	31
GA 22	TNTC	7	3	0	172	1	0	182	37	0	0	25	34
AH 8	TNTC	72	0	0	0	0	0	34	21	0	0	22	14
8	1	0	0	4	3	0	8	0	17	0	0	8	5
AH 9	TNTC	24	0	0	197	0	0	99	38	0	0	45	33
GA 12	139	9	0	0	30	0	0	0	0	0	0	4	7
Ag 10	15	9	0	0	0	0	0	0	0	0	0	60	95
GA 3	185	8	0	0	0	0	0	0	0	0	0	0	3
GA 64	112	13	0	0	0	0	0	0	0	0	0	1	0
Cg 1	16	0	0	0	0	0	0	0	18	0	0	0	41
Cg 2	31	0	0	0	0	0	0	0	0	0	0	35	0
PA-1	11	TNTC	—	TNTC	18	TNTC	TNTC	—	TNTC	—	—	97	232

"TNTC" — too numerous to count, more than 300 colonies.

" — " — the area treated with phage showed confluent growth.

produced no distinct colonies when treated with phage grown on PA-1. The area treated with phage showed thin, confluent growth.

The major question concerning these transduction data was that many reciprocal crosses did not agree; for example, see the reciprocal crosses between GA 73, AH 3 and GA 22. The transductions were all repeated several times with different phage preparations to be sure that the numbers were correct. There are poorly understood differences between mutants which cause some to function as better recipients or donors than others. Apparently, some regions of the genome may be more frequently transferred than others by some phages (Hayes, 1968). In a strictly quantitative transduction, these differences may be normalized but this is not possible with the method used in the present study, which is only semi-quantitative. Another reason for failure to act as donor or recipient for certain loci may be the presence of large deletions. Therefore, it was important to examine the transduction data to determine whether any of the mutants were incapable of participating in a genetic cross as either donor or recipient.

From the data shown in Table V, the mutants were divided into four groups according to the numbers of transductants in any cross. The mutants which behaved as good donors, i.e., produced more than 30 transductants in at least two crosses with other mutants were AJ 4, AH 3, GA 22, AH 8, AH 9, GA 12, Ag 10 and Cg 2; good recipients, based on the same criterion, were GA 73, AH 8, GA 12, Ag 10, Cg 1 and Cg 2; poor donor mutants were GA 73, 8, GA 3, GA 64 and Cg 1; and poor recipient mutants were AJ 4, AH 3, GA 22, 8, AH 9, GA 3 and GA 64.

The data have been rearranged in smaller groupings to show crosses within and between these groups. The numbers of transductants in

crosses between poor recipients were small as shown in Table VI and, with a few exceptions, crosses using any of these mutants except AJ 4 as recipient resulted in no transduction. AJ 4 was a better recipient than donor in these crosses. Taking the data for the other mutants as shown in the Table VI, one might conclude that these represent identical or closely linked loci. However, the enzyme data shown in Tables II and III indicate that the group is not homogeneous with respect to enzymatic defects. Another possible conclusion would be that transduction may not occur between these mutants; i.e., that they may be incapable of participating in a genetic cross.

Similar data for poor donors are shown in Table VII. The numbers of transductants in these crosses between poor donors were again quite low except for those involving GA 73 as recipient.

Results of crosses in which poor donors were used as recipients and poor recipients as donors are shown in Table VIII. For the three mutants which were both poor donors and poor recipients, i.e., 8, GA 3 and GA 64, no transduction occurred in a cross with any of the donors. Two of these mutants, GA 3 and GA 64, were also among the four which gave anomalous results with the wild type as donor. The major point of interest in Tables VI, VII, and VIII is that none of the mutants is completely incapable of acting as either donor or recipient. Therefore, it is most unlikely that a large deletion is responsible for low frequency of participation in transduction. It does appear that mutants 8, GA 3 and GA 64 are atypical in genetic crosses for a reason which is not known at present.

TABLE VI

CROSSES BETWEEN POOR RECIPIENTS

Donor	Recipient						
	AJ 4	AH 3	GA 22	8	AH 9	GA 3	GA 64
AJ 4	0	0	0	0	0	0	0
AH 3	4	0	0	0	0	0	0
GA 22	7	3	0	1	0	0	0
8	0	0	0	0	8	0	0
AH 9	24	0	0	0	0	0	0
GA 3	8	0	0	0	0	0	0
GA 64	13	0	0	0	0	0	0

TABLE VII

CROSSES BETWEEN POOR DONORS

Donor	Recipient				
	GA 73	8	GA 3	GA 64	Cg 1
GA 73	0	0	0	0	6
8	1	0	0	0	8
GA 3	185	0	0	0	0
GA 64	112	0	0	0	1
Cg 1	16	0	0	0	0

TABLE VIII

CROSSES USING POOR DONORS AS RECIPIENTS
AND POOR RECIPIENTS AS DONORS

Donor	Recipient				
	GA 73	8	GA 3	GA 64	Cg 1
AJ 4	132	0	0	0	0
AH 3	TNTC	0	0	0	20
GA 22	TNTC	1	0	0	25
8	1	0	0	0	8
AH 9	TNTC	0	0	0	45
GA 3	185	0	0	0	0
GA 64	112	0	0	0	1

CHAPTER IV

DISCUSSION

In this investigation, thirteen mutants unable to utilize glycerol as a carbon source for growth were used for enzymatic and genetic studies, and evidence was obtained for a previously unreported pathway of glycerol metabolism in P. aeruginosa.

Glycerol catabolism in P. aeruginosa was first studied by Cowen (1968). He found that the enzymes glycerol kinase and L-α-glycerophosphate dehydrogenase are involved in the glycerol catabolic pathway of P. aeruginosa (Figure 2, reactions 1 and 2). Brown (1972) found that a mutant (GA 73) lacking glycerol kinase produced large amounts of $^{14}CO_2$ from ^{14}C-glycerol. This indicated that P. aeruginosa must have an additional route for metabolism of glycerol. Brown (1972) detected, in a fresh PA-1 extract, a new enzyme activity, NADP-linked glycerol dehydrogenase, which produced D-glyceraldehyde. She suggested that this might be the initial reaction in an alternate metabolic route.

From D-glyceraldehyde there are two possible routes for further metabolism. One involves the enzyme D-glyceraldehyde dehydrogenase which would convert D-glyceraldehyde to D-glycerate. This enzyme was sought in the present study and was found to be present, at a level similar to the catabolic enzyme activities, in PA-1 as well as in all thirteen mutants. It was shown to be not linked to either NAD or NADP.

The other possible route for further metabolism of D-glyceraldehyde

would involve the enzyme D-glyceraldehyde kinase which would form glyceraldehyde-3-phosphate and would thus connect the new pathway to the Embden-Meyerhof-Parnas pathway for further catabolism since P. aeruginosa has a highly active triosephosphate isomerase (Cowen, 1968; Heath, 1971). No glyceraldehyde kinase activity was detected in the present study in an assay system which would have measured reduction of the product had it been formed. However, enzyme assays for D-glyceraldehyde dehydrogenase and glycerol dehydrogenase confirm a new pathway for glycerol metabolism. Glycerol dehydrogenase and D-glyceraldehyde dehydrogenase seem to be used only for lipid synthesis. This agrees with the fact that mutants like GA 73 and AJ 4 which lack glycerol kinase or L-α-glycerophosphate dehydrogenase cannot grow on glycerol. If these mutants, which do have glycerol dehydrogenase and D-glyceraldehyde dehydrogenase activities, also had glyceraldehyde kinase, or glycerate kinase, they should be able to grow on glycerol using the alternate pathway.

In addition, the new pathway may be the sole, obligatory pathway for synthesis of phosphatidylethanolamine from glycerol, since mutant GA 22, which lacks only glycerol dehydrogenase, cannot grow normally on glycerol. It is perhaps significant that growth on glycerol is not completely absent in this mutant (see Table I) but is very slow compared to the wild type. This may be due to a very severe limitation in formation of phospholipids for membrane synthesis.

The pathway involving glycerol and D-glyceraldehyde dehydrogenases is apparently unique among bacteria, or at least among those in which glycerol metabolism has been studied. The glycerol dehydrogenases reported in other bacteria oxidize the second carbon of glycerol to

form dihydroxyacetone. Kistler and Lin (1971) have recently discussed the functions of the various dehydrogenases which are related to glycerol metabolism in E. coli and A. aerogenes. In A. aerogenes, which can grow anaerobically with glycerol as sole carbon source, there are two dehydrogenases, a pyridine nucleotide-independent L-α-glycerophosphate dehydrogenase which functions during aerobic growth and an NAD-linked glycerol dehydrogenase which functions anaerobically to form dihydroxyacetone. In E. coli, which can use glycerol anaerobically only with an external terminal electron acceptor (nitrate or fumarate), there are two distinct L-α-glycerophosphate dehydrogenases which are not linked to pyridine nucleotide, and in addition there is an NAD-linked glycerol dehydrogenase, which converts glycerol to dihydroxyacetone, and an NADP-linked L-α-glycerophosphate dehydrogenase. The particulate (membrane-associated) L-α-glycerophosphate dehydrogenase can function either aerobically or anaerobically with nitrate and the physiological electron carrier for the reaction is not known. The soluble L-α-glycerophosphate dehydrogenase functions only under anaerobic conditions and is flavin-linked. Kistler and Lin (1971) have postulated that in E. coli the pyridine nucleotide-linked dehydrogenases do not function catabolically but are used for synthesis of glycerol or L-α-glycerophosphate.

In two strict aerobes, which can utilize glycerol only under aerobic conditions, the enzymes reported do not involve glyceraldehyde formation. In Mycob. tuberculosis, the initial enzyme in the catabolic pathway for glycerol is a dehydrogenase which, like the one detected in our studies, is linked to NADP. However, the product formed was identified as dihydroxyacetone and a dihydroxyacetone kinase was also

reported in this organism (Goldman, 1963). No dihydroxyacetone kinase activity has detected in our strain of P. aeruginosa (Cowen, 1968). In Acetobacter suboxydans, an organism closely related to P. aeruginosa, a completely different mode of glycerol metabolism has been reported (Cheldelin, 1961). There are two alternate reaction sequences for conversion of glycerol to dihydroxyacetone phosphate, as in the enterics, but the dehydrogenases have cofactor requirements which differ from those of the enterics, Mycob. tuberculosis, or P. aeruginosa. The glycerol dehydrogenase, which forms dihydroxyacetone, is pyridine-nucleotide-independent, while the L-α-glycerophosphate dehydrogenase of the alternate reaction sequence is linked to NAD. Thus, it appears that the metabolism of glycerol in bacteria may involve a very great variety of dehydrogenases which differ in cofactor requirement and in physiological function. The latter has not generally been determined. The pathway represented by the NADP-linked glycerol dehydrogenase and D-glyceraldehyde dehydrogenase, found in P. aeruginosa, has not been previously reported in bacteria to our knowledge. This pathway apparently represents a unique mechanism by which glycerol can be utilized for biosynthesis but not for catabolism.

Since the two new enzymes allow utilization of glycerol by an alternate route, assays for these enzymes as well as for the known catabolic enzymes and the active transport system were carried out on all mutants included in the present study. The importance of concurrent enzymatic and genetic studies is related to models for metabolic control. The operon model, as originally conceived, involves contiguous locations, i.e., clusters, of the structural genes for a pathway and control by promoter and operator genes which are also part of the cluster.

Although a number of exceptions to this type of genetic organization have now been reported, it has been estimated that approximately 72 percent of the related gene loci in Salmonella typhimurium occur in the clustering pattern predicted by the operon model and that similar clustering is probably as frequent in E. coli (Demerec, 1964).

In their early studies, Holloway, et al. (1963) had reported that Pseudomonas shows a different pattern of gene distribution from that found in E. coli and S. typhimurium. Later studies of Fargie and Holloway (1965), and studies of others (reviewed by Holloway, 1969) have shown that the related genes in any one pathway are often arranged in several clusters each consisting of only two or three genes, and in several cases genes within these clusters appear to be contiguous. It should perhaps be noted that mapping techniques in P. aeruginosa are not as advanced as in the enteric organisms.

All thirteen mutants and the wild type, PA-1, were induced with glycerol and cell-free extracts were assayed. None of the mutants lacked the enzyme glyceraldehyde dehydrogenase, and the activities of this enzyme were quite similar in all extracts assayed. Most of the mutants were deficient in one or more of the other three enzymes. The mutants were separated into several groups based on analysis of enzyme activities (Table III). Mutants with similar enzyme profiles were placed in the same group.

Several mutants had multiple enzyme lesions. Such mutations may be the result of large deletions of several structural genes or of single mutations with pleiotropic effects. The latter may be polar mutations or mutations in one of the regulatory genes, i.e., the regulator gene, the promoter or the operator. None of the mutants with

multiple defects can be explained as a deletion of all the structural genes involved, since each is capable of acting as donor for one or more of the structural genes in which it is non-functional. Therefore, all are apparently mutant in a regulatory locus or have nonsense or frame-shift mutations leading to polarity of gene expression. Comparison of enzymic and genetic data can yield information as to the nature of the mutations, based on known pleiotropic effects of such mutations in well-studied systems such as the lac operon.

AH 9 and GA 12 had essentially no glycerol dehydrogenase or glycerol kinase activity and had low L-α-glycerophosphate dehydrogenase activity; mutant 8 had low activity in all three enzymes. Therefore, AH 9, GA 12, and 8 appear to be promoter mutants, since mutations in the promoter region may lower the rate of synthesis of all the enzymes of the operon. Mutant 8 was less severely affected and was able to grow very slowly on glycerol (Table I). Ag 10, GA 3, and GA 64 lack glycerol kinase, L-α-glycerophosphate dehydrogenase and glycerol dehydrogenase. No activity was detected for any of the three enzymes. These mutants are similar to the non-inducible mutants of the lac operon in E. coli and could have a mutation in the regulator gene, resulting in production of a "super repressor" (R^S type mutation) which would prevent synthesis of any enzyme sensitive to that repressor. The mutants which have a single enzymatic defect, presumably in the structural gene, include AJ 4 and AH 3, which lack L-α-glycerophosphate dehydrogenase, GA 22, which lacks glycerol dehydrogenase, and GA 73, which lacks glycerol kinase. The mutants Cg 1 and Cg 2 grew very slowly in both glycerol and glucose minimal media but grew normally with lactate, succinate or yeast extract as carbon source. These

characteristics are identical to those of a number of glucose-negative mutants studied by Heath (1971), who suggested that these mutants might be unable to grow on glycerol because of a requirement for induction of the catabolic glyceraldehyde-3-phosphate dehydrogenase by glucose-6-phosphate. Therefore, it is probable that mutants Cg 1 and Cg 2 have mutations which prevent conversion of glycerol to glucose-6-phosphate (Figure 1) and are unable to grow on glycerol as an indirect result of a mutation outside the specific pathways for glycerol. They, like the mutants studied by Heath, were not deficient in either of the catabolic enzymes for glycerol, nor did they lack either of the enzymes for conversion of glycerol to glycerate.

While the method of transduction used in the present study is only semi-quantitative, the numbers of transductants obtained in crosses performed simultaneously should have meaning as relative values and have been used in this way in transductional analysis in P. aeruginosa. Thus, the data may be used as a basis for tentative conclusions regarding linkage of the loci and, if linked, of their relative positions.

The usual test for linkage of related loci by transduction, in cases where transfer of specific loci cannot be detected by selection, is comparison of numbers of transductants obtained in crosses between wild type and mutant with those for crosses between mutants. Transfer of linked markers, one of which is mutant, decreases the probability of recombination to yield a wild type transductant and thus decreases the total number of transductants, which are selected for the wild type phenotype. Therefore, if significantly higher numbers are obtained using the wild type as donor for two mutants than are obtained in a

cross in either direction between those two mutants, this is considered evidence for linkage. This type of data was used by Holloway, et al. (1963) and Fargie and Holloway (1965) in their studies of linkage of related loci in P. aeruginosa.

Inspection of Table V shows that crosses of mutants AJ 4, GA 22, 8, AH 9, and Ag 10 with the wild type yielded much higher numbers of recombinants than did crosses with any other mutant. These represent loci for the L-α-glycerophosphate dehydrogenase and the glycerol dehydrogenase and, according to the analysis of enzyme data given above, the loci for the promoter and the regulator gene. Therefore, these loci appear to be linked. Crosses of the wild type with the other mutants produced anomalous results. The crosses with AH 3, GA 12, GA 3 and GA 64 have been discussed previously. Mutants GA 73 and AH 8 were obtained from Dr. Karen Brown, who had used them over a period of two years during which they had been transferred and repurified as single colony isolates repeatedly. It is believed that they are no longer isogenic with the wild type strain used in these studies (Curtiss, 1969). Otherwise, the minimum number of transductants obtained in wild type crosses should have been as large as the number obtained with any mutant. This inconsistency in the behavior of GA 73 as a recipient means that no genetic data relative to linkage of the locus for glycerol kinase to other glycerol loci can be obtained. However, if the hypothesis that AH 9, 8 and GA 12 are promoter mutants is correct, the kinase locus must belong to the same cluster as the two dehydrogenase loci. The finding that the kinase is included in the pleiotropic effects of the mutations in these three strains argues for linkage of this locus to the others.

The number of transductants from crosses among related gene pairs (Table IX) may suggest the relative positions of these genes. Crosses of GA 73 (GK) with mutants in any other gene yield the highest numbers of transductants, which indicates that the glycerol kinase gene should be farthest from the other loci. The numbers of transductants from a glycerol dehydrogenase mutant crossed with either regulator or promoter mutants are larger than the number from an L-α-glycerophosphate dehydrogenase mutant crossed with either regulator or promoter mutants. This indicates that the glycerol dehydrogenase gene is farther from the regulator or promoter than is the L-α-glycerophosphate dehydrogenase gene. These two genes produced only a few recombinants, indicating definite clustering. The crosses of regulator mutants with an L-α-glycerophosphate dehydrogenase mutant also produced a low number of transductants. This result indicates a similarity to E. coli where Cozzarelli, et al. (1968) and Berman-Kurtz, et al. (1971) reported that the aerobic L-α-glycerophosphate dehydrogenase locus was adjacent to that for the regulator gene. It seems that a close linkage may also occur in P. aeruginosa.

The mutant AH 8 has no glycerol dehydrogenase or L-α-glycerophosphate dehydrogenase activity, and low glycerol transport. This mutant could be a promoter or polarity mutant. If AH 9 and GA 12 which have no glycerol kinase, no L-α-glycerophosphate dehydrogenase, low glycerol dehydrogenase and low transport are promoter mutants, then AH 8 may not be a promoter mutant since its pleiotropic effect does not include the kinase. It is possible that AH 8 has a deletion in the region between two of the structural genes with a polar effect on the third, or it could be a double mutant with a deletion affecting

TABLE IX

TRANSDUCTION AMONG RELATED GENE GROUPS

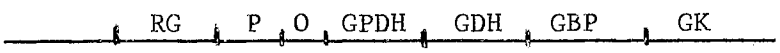
Gene pair	Maximum number transductants
RG-Pro	38
RG-GPDH	13
RG-GDH	37
RG-GK	185
Pro-GPDH	97
Pro-GDH	182
Pro-GK	TNTC
GPDH-GDH	7
GPDH-GK	TNTC
GDH-GK	TNTC

Abbreviations:

RG-Regulator gene
Pro-Promoter gene
GDH-Glycerol dehydrogenase
GPDH-L-α-glycerophosphate dehydrogenase
GK-Glycerol kinase

two adjacent structure genes and a second mutation in the third gene. Brown (1972) demonstrated that the loss of specific glycerol binding protein was the reason for the low glycerol transport in AH 8. A deletion in the region between L-α-glycerophosphate dehydrogenase and glycerol dehydrogenase could possibly affect the gene for the glycerol binding protein if it were contiguous to these two genes. In this case, the structural genes for L-α-glycerophosphate dehydrogenase, glycerol dehydrogenase and the glycerol binding protein might be expected to be contiguous, with a single operator and promoter. The structural gene for glycerol kinase is controlled by the same regulator. This conclusion is based on parallel induction of kinase and dehydrogenase (Tsay, 1971; Brown, 1972) and is consistent with pleiotropic effects of the postulated regulator mutation in Ag 10, GA 3 and GA 64. However, the generally large numbers of transductants produced in crosses of GA 73 and all other mutants indicate that the kinase locus is somewhat distant from the other loci. Therefore, it seems likely that the gene for the glycerol binding protein (GBP) is between the loci for glycerol dehydrogenase and glycerol kinase.

From the above analysis a possible map could be drawn as follows:



The map positions of the loci for the three structural genes are based on the data shown in Table IX. It should be noted that crosses involving each of these loci with the regulator locus and the promoter locus agree; i.e., the relative numbers of transductants obtained in the two sets of crosses increase in the order GPDH-GDH-GK. All the

data indicate that the structural genes are more closely linked to the regulator gene than to the promoter, but this would be contrary to accepted theories of control. Therefore, the regulator and the operator have been assumed to be in the same positions relative to the promoter as in the lac operon. Further study of the glycerol operon with additional mutants will be needed to confirm this tentative map.

The data on transport have not been considered in characterization or mapping of the mutants except for AH 8 in which the nature of the transport defect is known. Active transport can be affected by several different mutations since several components are involved in the transport system. Several different proteins may be capable of binding glycerol in P. aeruginosa (Brown, 1972). Loss of transport activity could also be caused by a defect in the energy linkage. Brown (1972) speculated that transport of glycerol in P. aeruginosa may be linked to one or more dehydrogenases as in the model proposed by Barnes and Kaback (1971). It is interesting to note that loss of either glycerol dehydrogenase or L-α-glycerophosphate dehydrogenase does not necessarily affect transport since uptake of glycerol was normal in mutants GA 22 and AJ 4. Tsay (1971) found that transport of glycerol requires a binding protein inducible by glycerol and absent in a transport-negative mutant (Tsay, et al., 1971). The studies by Brown (1972) also showed that P. aeruginosa has an active transport system for glycerol. Without extensive studies of binding proteins and mutants specifically defective in transport, it is impossible to determine the reason for low transport activity or location of the transport gene (or genes). However, the present study adds further support to the conclusion that transport of glycerol in P. aeruginosa is not dependent upon the activity of any of the enzymes involved in glycerol metabolism.

CHAPTER V

SUMMARY AND CONCLUSIONS

Thirteen glycerol-negative mutants were used in this study. All these mutants can grow normally in glucose, succinate and lactate minimal medium except Cg 1 and Cg 2, which cannot grow on either glycerol or glucose. The primary genetic lesion in these mutants was concluded to be in glucose metabolism since they seem identical to glucose-negative mutants previously studied.

The glycerol catabolic pathway in P. aeruginosa was studied through glycerol-negative mutants. This pathway is catalyzed by glycerol kinase and L-α-glycerophosphate dehydrogenase. A recently reported glycerol dehydrogenase (Brown, 1972) was also studied. All these enzyme activities were detected in the wild type, PA-1, but one or more of the enzyme activities was defective in each mutant used in these studies. Three mutants, Ag 10, GA 3, and GA 64 completely lack all three enzyme activities and are thought to be regulator gene mutants of the super repressor type. AH 9, GA 12 and 8 appear to be promoter mutants. The mutants AJ 4 and AH 3 lack L-α-glycerophosphate dehydrogenase, GA 22 lacks glycerol dehydrogenase, and GA 73 lacks glycerol kinase. These mutants apparently have single mutations in the corresponding structural genes. AH 8 lacks glycerol dehydrogenase, L-α-glycerophosphate dehydrogenase and the glycerol binding protein; it may be a polarity mutant.

The transduction studies showed that the genes coding for these three enzymes are probably linked, with the L-α-glycerophosphate dehydrogenase gene lying nearest to the regulator (or promoter) gene. The genes coding for L-α-glycerophosphate dehydrogenase and glycerol dehydrogenase seem to be contiguous, or clustered together. The glycerol kinase gene is farthest from the regulator (promoter) gene and appears not to be contiguous to the other two structural genes because high numbers of transductants were produced with other mutants. However, these three structural genes were controlled by the same regulator gene. Based on analysis of both the transduction data and the enzyme activities, a tentative genetic map was presented.

An alternate pathway of glycerol metabolism, which has not been reported previously in bacteria, was found in this study. A new dehydrogenase, D-glyceraldehyde dehydrogenase, was found to be pyridine-nucleotide independent. The product is assumed to be D-glycerate. This activity was detected in PA-1 as well as in all mutants. Therefore, it was concluded that P. aeruginosa possesses a pathway by which glycerol can be converted to D-glyceraldehyde, then to D-glycerate and probably eventually to phosphatidylethanolamine through hydroxypyruvate. This may be a major pathway leading to phospholipid biosynthesis. These reactions apparently have no function in glycerol catabolism. No glyceraldehyde kinase activity was detected in cell extracts and loss of one of the catabolic enzymes prevents growth on glycerol.

The transport of glycerol was shown to occur at normal levels in some mutants (GA 73, AJ 4, GA 22, 8, Cg 1, Cg 2) and at low levels in others (AH 3, AH 8, GA 12, GA 64, AH 9). No correlation was found

between a defect in any enzyme and lowered ability to transport glycerol. This supports the conclusion that there is a specific transport system for glycerol in P. aeruginosa, which is not dependent upon the activity of any of the enzymes involved in metabolism of glycerol.

BIBLIOGRAPHY

Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in Escherichia coli K12. Biochem. Biophys. Res. Commun. 18: 788-795.

Asnis, R. E., and A F. Brodie. 1953. A glycerol dehydrogenase from Escherichia coli. J. Biol. Chem. 203: 153-159.

Barnes, E. M., Jr., and H. R. Kaback. 1971. Mechanisms of active transport in isolated membrane vesicles. I. The site of energy coupling between D-lactic dehydrogenase and β-galactoside transport in Escherichia coli membrane vesicles. J. Biol. Chem. 246: 5518-5522.

Beckwith, J. R., and D. Zipser. 1970. The lactose operon. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Berman-Kurtz, M., E. C. C. Lin, and D. P. Richey. 1971. Promoter-like mutant with increased expression of the glycerol kinase operon of Escherichia coli. J. Bacteriol. 106: 724-731.

Brown, K. K. 1972. Characterization of binding and transport of glycerol by Pseudomonas aeruginosa. Ph.D. Thesis, Oklahoma State University, Stillwater.

Burton, R. M., and N. O. Kaplan. 1953. A DPN specific glycerol dehydrogenase from Aerobacter aerogenes. J. Am. Chem. Soc. 75: 1005-1006.

Chakrabarty, A. M., C. F. Gunsalus, and I. C. Gunsalus. 1968. Transduction and the clustering of genes in fluorescent pseudomonads. Proc. Natl. Acad. Sci. U.S.A. 68: 168-175.

Cheldelin, V. H. 1961. Metabolic pathways in microorganisms. John Wiley and Sons, New York.

Cozzarlli, N. R., W. B. Freedberg, and E. C. C. Lin. 1968. Genetic control of the L-α-glycerophosphate system in Escherichia coli. J. Mol. Biol. 31: 371-387.

Cowen, C. M. 1968. Catabolic pathways and metabolic controls in Pseudomonas aeruginosa. M.S. Thesis, Oklahoma State University, Stillwater.

Crawford, I. P., and I. C. Gunsalus. 1966. Inducibility of tryptophan synthetase in Pseudomonas putida. Proc. Natl. Acad. Sci. U.S.A. 56: 717-724.

Curtiss, R. 1969. Bacterial conjugation. Ann. Rev. Microbiol. 23: 69-136.

Dawson, R. M., D. C. Elliott, W. H. Elliott, and K. M. Jones. 1959. Data for biochemical research. Oxford University Press, London.

De Crombrugghe, B., B. Chen, W. Anderson, P. Nissley, M. Gottesman, I. Pastan, and R. Perlman. 1971. Lac DNA, RNA polymerase and cyclic AMP recepter protein, cyclic AMP, lac repressor and inducer are the essential elements for controlled lac transcription. Nature New Biol. 231: 139-142.

Demerec, M. 1964. Clustering of functionally related genes in Salmonella typhimurium. Proc. Natl. Acad. Sci. U.S.A. 51: 1057-1060.

Epstein, W., and J. R. Beckwith. 1968. Regulation of gene expression. Ann. Rev. Biochem. 37: 411-436.

Fargie, B., and B. W. Holloway. 1965. Absence of clustering of functionally related genes in Pseudomonas aeruginosa. Genet. Res. 6: 284-299.

Goldman, D. S. 1963. Enzyme systems in the Mycobacteria. XV. Initial steps in the metabolism of glycerol. J. Bacteriol. 86: 30-37.

Gregory, J. D., and S. W. Sajdera. 1970. Interference in the Lowry method for protein determination. Science 169: 97-98.

Gunsalus, I. C. 1947. Products of anaerobic glycerol fermentation by Streptococcus faecalis. J. Bacteriol. 54: 239-244.

Gunsalus, I. C., C. F. Gunsalus, A. M. Chakrabarty, S. Sikes, and I. P. Crawford. 1968. Fine structure mapping of the tryptophan genes in Pseudomonas putida. Genetics 60: 419-435.

Gunsalus, I. C., and W. W. Umbreit. 1945. The oxidation of glycerol by Streptococcus faecalis. J. Bacteriol. 49: 347-357.

Hayashi, S., and E. C. C. Lin. 1965. Capture of glycerol by cells of Escherichia coli. Biochem. Biophys. Acta. 94: 479-487.

Hayes, W. 1968. The genetics of bacteria and their viruses. John Wiley and Sons, Inc., New York.

Heath, H. E. 1971. Glucose metabolism and catabolite repression in Pseudomonas aeruginosa. Ph.D. Thesis, Oklahoma State University, Stillwater.

Holloway, B. W. 1969. Genetics of Pseudomonas. Bacteriol. Rev. 33: 419-443.

Holloway, B. W., L. Hodgins, and B. Fargie. 1963. Unlinked loci affecting related biosynthetic steps in Pseudomonas aeruginosa. Nature 199: 926-927.

Holloway, B. W., V. Krishnapillai, and V. Stanisich. 1971. Pseudomonas genetics. Ann. Rev. Genet. 5: 425-441.

Holloway, B. W., M. Monk, L. Hodgins, and B. Fargie. 1962. Effects of radiation on transduction in Pseudomonas aeruginosa. Virology 18: 89-94.

Hunter, G. J. E. 1953. The oxidation of glycerol by Mycobacteria. Biochem. J. 55: 320-328.

Ippen, K., J. H. Miller, J. G. Scaife, and J. R. Beckwith. 1968. New controlling element in the lac operon for E. coli. Nature 217: 825-827.

Jacob, F., and J. Monod. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. 3: 318-356.

Jacob, F., A. Ullman, and J. Monod. 1964. Le promoteur, element genetique necessaire a l'expression d'un operon. Compt. Rend. Acad. Sci. 258: 3125-3128.

Jacobs, N. J. and P. J. Van Demark. 1960. Comparison of the mechanism of glycerol oxidation in aerobically and anaerobically grown Streptococcus faecalis. J. Bacteriol. 79: 532-538.

Kistler, W. S., and E. C. C. Lin. 1971. Anaerobic L-α-glycerophosphate dehydrogenase of E. coli: Its genetic locus and its physiological role. J. Bacteriol. 108: 1224-1234.

Koch, J. P., S. I. Hayashi, and E. C. C. Lin. 1964. The control of dissimilation of glycerol and L-α-glycerophosphate in Escherichia coli. J. Biol. Chem. 239: 3106-3108.

Lin, E. C. C., A. P. Levin, and B. Magasanik. 1960. The effect of aerobic metabolism on the inducible glycerol dehydrogenase of Aerobacter aerogenes. J. Biol. Chem. 235: 1824-1829.

Lin, E. C. C., J. P. Koch, T. M. Chused, and S. E. Jorgensen. 1962. Utilization of L-α-glycerophosphate by Escherichia coli without hydrolysis. Proc. Natl. Acad. Sci. U.S.A. 48: 2145-2150.

Magasanik, B., M. S. Brooke, and D. Karibian. 1953. Metabolic pathway of glycerol dissimilation. J. Bacteriol. 66: 611-619.

Marinus, M. G., and J. S. Loutit. 1969a. Regulation of isoleucine-valine biosynthesis in Pseudomonas aeruginosa. I. Characterisation and mapping of mutants. Genetics 63: 547-556.

Marinus, M. G., and J. S. Loutit. 1969b. Regulation of isoleucine-valine biosynthesis in Pseudomonas aeruginosa. II. Regulation of enzyme activity and synthesis. Genetics. 63: 557-567.

Martin, R. G. 1969. Control of gene expression. Ann. Rev. Genet. 3: 181-216.

Mickelson, M. N., and F. E. Shideman. 1947. The oxidation of glycerol by Escherichia freundii. Arch. Biochem. 13: 437-448.

Miller, J. H., K. Ippen, J. G. Scaife, and J. R. Beckwith. 1968. The promoter-operator region of the lac operon of Escherichia coli. J. Mol. Biol. 38: 413-420.

Mindich, L. 1968. Pathway for oxidative dissimilation of glycerol by Bacillus subtilis. J. Bacteriol. 96: 565-566.

Murphy, W. H., and E. D. Rosenblum. 1964. Selective medium for carbohydrate-utilizing transductants of Staphylococcus aureus. J. Bacteriol. 87: 1198-1201.

Pastan, I., and R. L. Perlman. 1968. The role of the lac promoter locus in the regulation of β-galactosidase synthesis by cyclic 3', 5'-AMP. Proc. Natl. Acad. Sci. U.S.A. 61: 1336-1342.

Perlman, R., and I. Pastan. 1968. Cyclic 3', 5'-AMP: Stimulation of β-galactosidase and tryptophanase induction in E. coli. Biochem. Biophys. Res. Commun. 30: 656-664.

Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten. 1957. Studies of biosynthesis in Escherichia coli, 2nd printing. Carnegie Inst. Wash. Pub. 607: 5.

Rush, D., D. Karibian, M. L. Karnovsky, and B. Magasanik. 1957. Pathways of glycerol dissimilation in two strains of Aerobacter aerogenes: enzymatic and tracer studies. J. Biol. Chem. 226: 891-899.

Sanno, Y., T. H. Wilson, and E. C. C. Lin. 1968. Control of permeation to glycerol in cells of Escherichia coli. Biochem. Biophys. Res. Commun. 32: 344-349.

Silverstone, A. E., B. Magasanik, W. S. Reznikoff, J. H. Miller, and J. R. Beckwith. 1969. Catabolite-sensitive site of the lac operon. Nature 221: 1012-1014.

Sowerby, J. M., and J. H. Ottaway. 1966. The enzymic estimation of glutamate and glutamine. Biochem. J. 99: 246-252.

Stanier, R. Y., N. J. Palleroni, and M. Douderoff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43: 159-271.

Sutherland, E. W., C. F. Cori, R. Haynes, and N. S. Olson, 1949. Purification of the hyperglycemic-glycogenolytic factor from insulin and from gastric mucosa. J. Biol. Chem. 180: 825-837.

Sutter, V. L., V. Hurst, and J. Fennell. 1963. Semisynthetic medium for bacteriophage typing of Pseudomonas. J. Bacteriol. 86: 1354-1356.

Swanstrom, M., and M. H. Adams. 1951. Agar layer method for production of high titer phage stocks. Proc. Soc. Exptl. Biol. Med. 78: 372-375.

Tsay, S. S. 1971. Transport and catabolism of glycerol in Pseudomonas aeruginosa. Ph.D. Thesis, Oklahoma State University, Stillwater.

Tsay, S. S., K. K. Brown, and E. T. Gaudy. 1971. Transport of glycerol by Pseudomonas aeruginosa. J. Bacteriol. 108: 82-88.

Virtanen, A. I., and M. Nordlund. 1933. An improved method for the preparation of dihydroxyacetone. J. Biochem. 27: 442-444.

White, F. E., Jr. 1972. Genetic studies in Pseudomonas aeruginosa. Ph.D. Thesis, Oklahoma State University, Stillwater.

Wiame, J. M., S. Bourgeois, and R. Lambion. 1954. Oxidative dissimilation of glycerol studied with variants of Bacillus subtilis. Nature 174: 37-38.

Winder, F. G., and P. J. Brennan. 1966. Initial steps in the metabolism of glycerol by Mycobacterium tuberculosis. J. Bacteriol. 92: 1846-1847.

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