METABOLIC DEFECTS AND PHAGE RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

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METABOLIC DEFECTS AND PHAGE RESISTANCE

IN PSEUDOMONAS AERUGINOSA

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CHAPTER I

INTRODUCTION

Overall Scope of Research

The aim of this study was to investigate the metabolic and genetic characteristics of a previously isolated group of mutants of Pseudomonas aeruginosa. These mutants had been isolated by Cowen (1) by treatment of the wild type P. aeruginosa, strain 1, with nitrosoguanidine as the mutagenic agent. They were chosen for further study because they seemed to possess unusual defects. Fifty-five organisms in this series all seemed to possess identical defects, so far as studied. They had been isolated originally as mutants unable to use glycerol, but they were also unable to grow on minimal medium containing glucose. This lack of ability to grow on glucose could be remedied by the addition of yeast extract, which allowed growth to a level limited by the amount of yeast extract added. Yet the addition of yeast extract failed to allow growth on glycerol. Also, the organisms had lost the ability to grow on mannitol.

Another interesting characteristic of these organisms was the complete absence of lysis on exposure of the cells to 13 different strains of P. aeruginosa phage. Thus phage resistance was also apparently related to the other mutational changes.

It was felt that these mutants warranted additional investigation in order to further characterize their defects, especially with respect

to the yeast extract requirement. Possibly other defects were also present that would be useful in further studies of metabolic pathways or control mechanisms. They might also be useful in genetic studies with Pseudomonas involving genetic control in an organism in which many related loci have been reported to be unlinked, since much of our knowledge of genetic control mechanisms has been obtained from Escherichia coli, in which related loci have been found to be linked in most cases. Such studies could prove of some value in discovering possible new control mechanisms since Pseudomonas belongs to a different family than that on which essentially all of our present concepts are based. Detailed knowledge of comparable mechanisms in the two unrelated families may be important in attempts to extrapolate facts and theories relevant to microbial systems to those of higher animals.

Previous Work Done In Areas Studied

Glycerol Degradation

Two principle pathways for the utilization of glycerol in bacteria are known (see Figure 1). In one pathway, glycerol is first phosphorylated to glycerophosphate before oxidation to dihydroxyacetone phosphate. In the other, glycerol is first oxidized to dihydroxyacetone, then phosphorylated to form dihydroxyacetone phosphate. The dihydroxyacetone phosphate so formed in either case is then converted into glyceraldehyde-3-phosphate and proceeds through the usual glycolytic scheme (Embden-Meyerhof-Parnas pathway). The first pathway has been demonstrated in Mycobacterium, Streptococcus faecalis, Escherichia freundii, and Aerobacter aerogenes (2), and also in E. coli (3). The second pathway has been found in Acetobacter suboxydans and A.

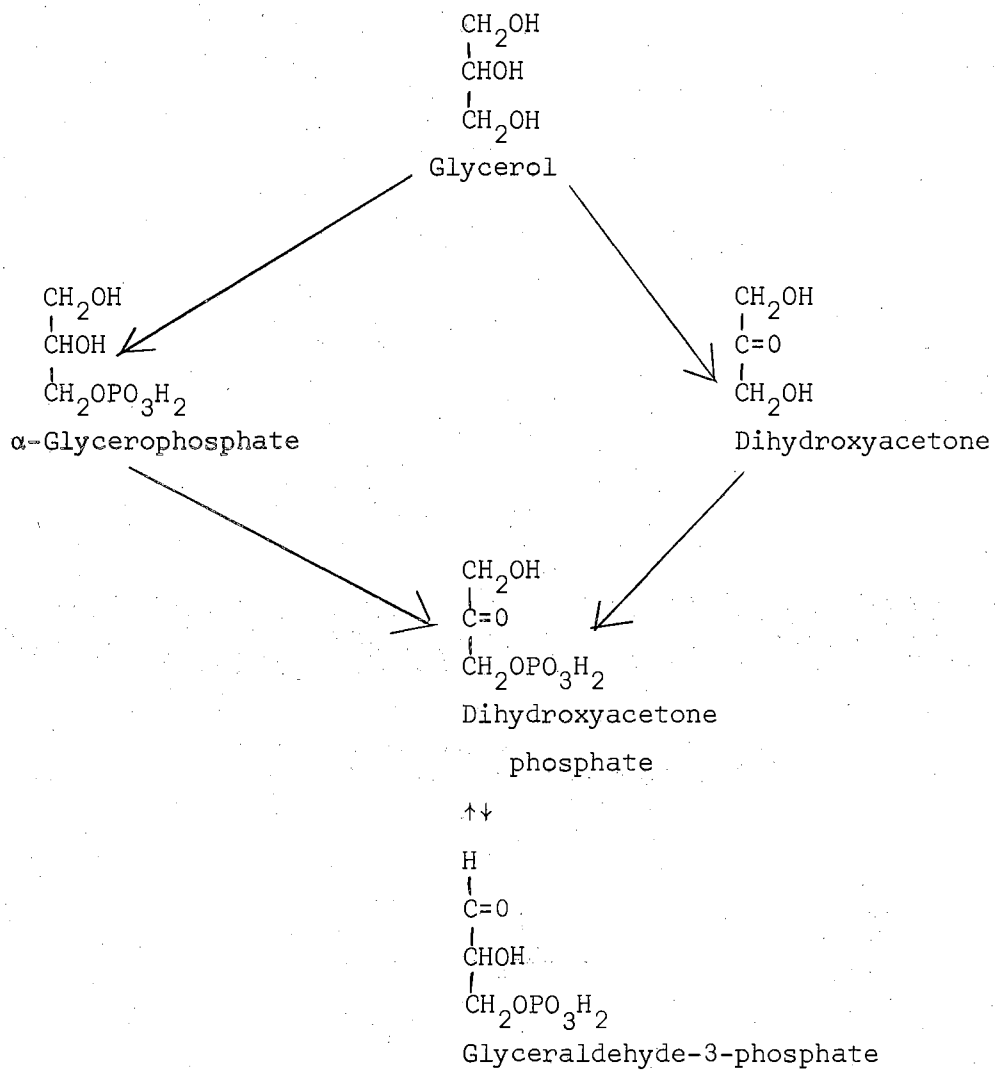


Figure 1. Major Pathways for Glycerol Metabolism in Bacteria

aerogenes (2), and also in E. coli (4). Lin et al. (5) have reported the occurence of both pathways in a strain of A. aerogenes. This organism contains an NAD-linked glycerol dehydrogenase which is induced by growth on glycerol under anaerobic conditions. A different glycerophosphate dehydrogenase, which does not require NAD, is active under aerobic conditions and converts glycerophosphate into dihydroxyacetone phosphate.

Different pathways have been described for E. coli by different investigators. Asnis and Brodie (4) reported that dihydroxyacetone was formed by extracts, but included no data on growth conditions of the cells. Koch et al. (3) reported more recently that the aerobic pathway proceeds via α-glycerophosphate, and that the anaerobic pathway is absent.

Cowen (1) has shown the pathway in P. aeruginosa to be the same as that found in E. coli by Koch et al. (3), involving glycerol kinase, a non-NAD-linked L-α-glycerophosphate dehydrogenase, and triose phosphate isomerase. However, he found no permease present in P. aeruginosa for α-glycerophosphate, as shown by the inability of cells to use this compound for growth. In this respect, P. aeruginosa differs from E. coli, in which a specific transport mechanism for uptake of L-α-glycerophosphate is induced by L-α-glycerophosphate (3,6).

Methionine Biosynthesis

The pathway for synthesis of methionine in E. coli is shown in Figure 2. Aspartic acid is converted to aspartic-β-semialdehyde and then to homoserine, which reacts with succinylcoenzyme A to form O-succinylhomoserine. The succinyl moiety is replaced by cysteine to

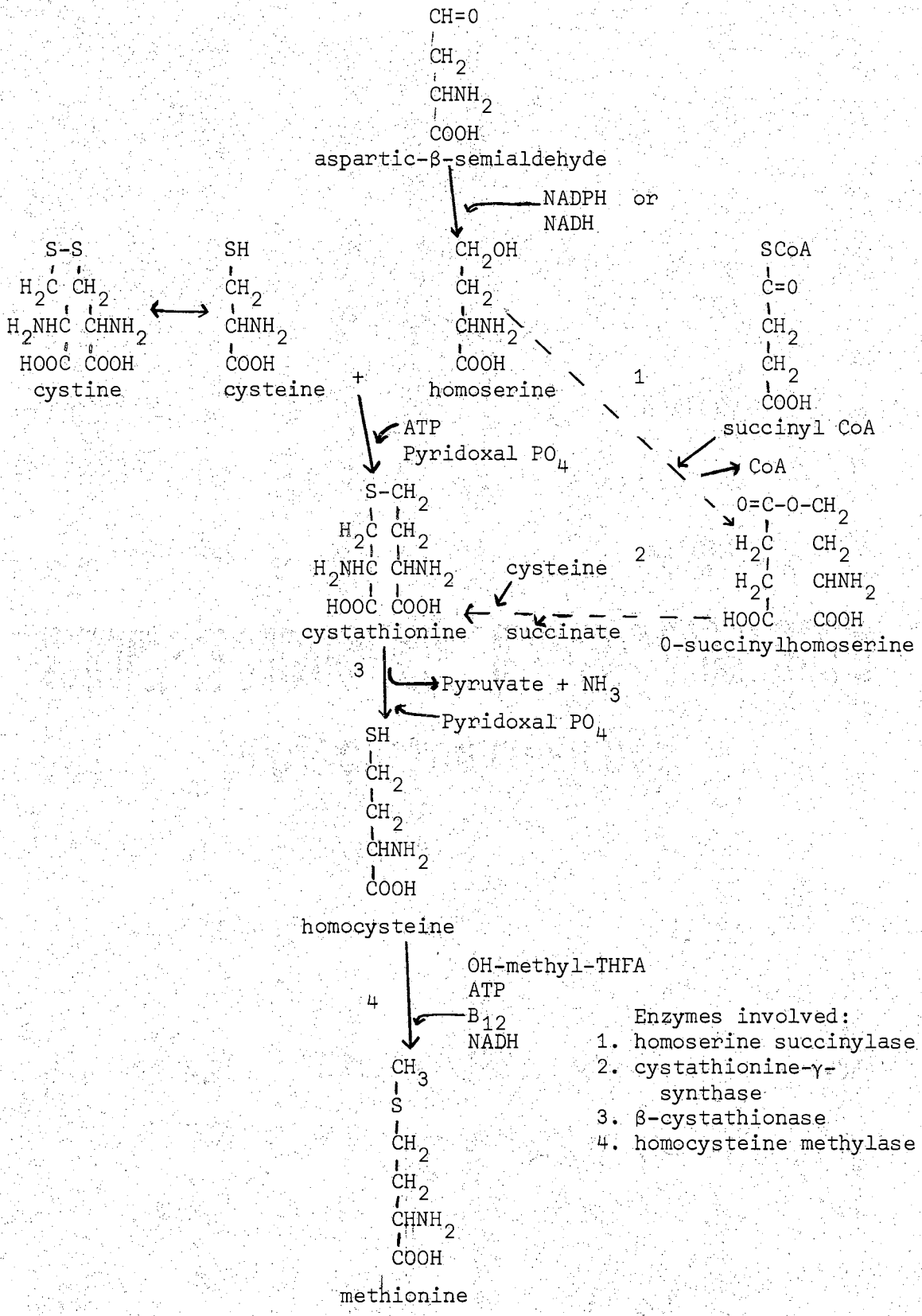


Figure 2. Methionine Pathway in Escherichia coli

form cystathionine. Cystathionine is then broken down into homocysteine, pyruvate, and NH_3, and homocysteine is converted to methionine.

The presence of O-succinylhomoserine as an intermediate in the conversion of homoserine to cystathionine has been known for a comparatively short length of time. In 1961, Rowbury (7) and Smith (8) indicated that more than one enzyme was concerend with the formation of cystathionine from homoserine and cysteine in both E. coli and Salmonella typhimurium. In 1964, Rowbury and Woods (9) presented strong evidence that O-succinylhomoserine was the intermediate formed in E. coli. It was also determined that N-succinylhomoserine would not function as the intermediate (10). O-succinylhomoserine was used much more readily than homoserine in the synthesis of homocysteine (11) (by the same set of reactions) by bacteria. In the case of yeast and Neurospora, acetylhomoserine replaces the O-succinylhomoserine found in bacteria (11,12,13), and these compounds may not be substituted for each other. Cystathionine has been confirmed as an intermediate in methionine biosynthesis in Neurospora also (14).

The enzyme β-cystathionase has been shown in E. coli to cleave cystathionine to form homocysteine and simultaneously to deaminate the three-carbon unit to form pyruvate and ammonia, one mole of each per mole of cystathionine, with the one enzyme responsible for all of these functions (15). Yeast and Neurospora have been shown capable of the above reaction and in addition they can cleave cystathionine into cysteine and α-ketobutyrate (16), but this second reaction, although present in mammals, has not been shown in bacteria and is considered to be absent.

Transductions and Genetic Studies With Pseudomonas

The two methods most generally used in genetic studies with bacteria are conjugation and transduction. Transduction is the method most frequently used in *P. aeruginosa* since no system has been worked out as completely as in *E. coli* for conjugation.

Since it was first described by Zinder and Lederberg (17), transduction has been a valuable genetic tool. Because only a small piece of DNA is involved in the transfer, transduction is very useful for studying short segments of the chromosome. For this reason it is the method of choice for studying the localized arrangement of the genes on the chromosome and their location with respect to one another and therefore is especially useful in determining linkage relationships for related loci.

The actual length of the DNA segment incorporated during transduction is not known, but in *E. coli* Lennox (18) has demonstrated cotransduction of up to four markers. Yura (19) showed that transductional analysis was capable of distinguishing groups of mutants with blocks in different reaction steps in a pathway. Transduction within a single strain of *P. aeruginosa* was reported in 1958 (20), and several workers have used transduction to group mutants of *P. aeruginosa* and to investigate linkage of related loci, sometimes in conjunction with growth and crossfeeding data.

After the isolation of phage F116 (21,22), and the demonstration that it was suitable for fine structure study of the *P. aeruginosa* chromosome (23), several studies were done. These included grouping of mutants with isoleucine-valine deficiencies (24), allantoin mutants (25),

acetate and acetamide mutants (26,27), histidine mutants (28,29), and methionine mutants (30). With few exceptions, most of the work agreed with reports of Holloway and co-workers (23,31) that related loci were unlinked in P. aeruginosa in the vast majority of cases. Many of these studies had insufficient data to characterize the total pathway or were not specifically designed to test for linkage between related markers. However, a few loci did appear to be linked: four loci controlling synthesis of isoleucine-valine (24), possibly two loci for methionine synthesis (30), two loci in the homoserine-threonine pathway and possibly two loci for the tryptophan pathway (31). These studies represented randomly selected mutants and did not include complete pathways. However, these findings seem to indicate that the initial reports of absence of linkage of related genes in P. aeruginosa (23,31) may have been premature. Recently, there has also been reported a study of the mandelate catabolic pathway, which indicates that nine loci in this pathway are linked (32).

From composite information obtained with conjugation, transduction, and biochemical studies, it has been indicated that E. coli, Sal. typhimurium, Bacillus subtilis, and Staphylococcus aureus all show clustering of loci affecting sequential steps in certain biosynthetic or degradative pathways. Loci involved in the tryptophan biosynthetic pathway (33,34), enzymes of the pyrimidine pathway (35), and the isoleucine-valine pathways (36) have been shown to be clustered in E. coli. In Sal. typhimurium, a high degree of linkage of functionally related genes is reported (37) for fourteen groups of genes, and this report is substantiated by other workers, who have found linkage in the histidine pathway (38), the tryptophan pathway (39), and the

leucine operon (40). In B. subtilis, linkage has been shown for markers in the tryptophan pathway (41), and a cluster of genes has been found for aromatic biosynthesis (42). The histidine region has been shown to exhibit linkage in Staph. aureus (43).

On the other hand, linkage has not been found to any significant degree in Neurospora crassa or Saccharomyces cerevisiae. The histidine biosynthetic pathway in both N. crassa (44, 45) and yeast (46) has been reported to lack linkage. These findings are thought to be significant in relation to the evolution of the operon control system (37), in the sense that this theory (47) is applicable to organisms with clustering of related genes.

Of further interest are the experiments of Isaac and Holloway (48), in which the pathway for synthesis of uracil was shown to be the same in both E. coli and P. aeruginosa. No linkage for four enzymes of the pathway was reported for P. aeruginosa, whereas they are linked in E. coli, and the control of enzyme synthesis in P. aeruginosa is unlike that in E. coli.

Cozzarelli and Lin (49) showed in 1966 that loci for methionine biosynthesis and glycerol kinase are closely linked in E. coli. No data on such loci are available for P. aeruginosa.

Modification and Restriction of DNA

Modification and restriction are two related processes by which a bacterial host can exert a strain-specific change in the DNA of a bacteriophage and can then either restrict or permit the multiplication of the phage within the host cell. Current information on modification and restriction has been reviewed recently (50,51). When phage DNA

multiplies inside the bacterial cell, the host bacterium imparts a strain-specific modification to the DNA. A particular bacterial strain can be successfully infected only with phage carrying the DNA modification produced by the strain. Infection with phage grown on other hosts is abortive and the unmodified DNA is broken down upon penetration into the host cells, these cells being restrictive for this DNA. This phenomenon has been observed with phage DNA's, including lambda, and with bacterial DNA.

Restriction results in endonucleolytic scission of DNA molecules, presumably at the specificity sites, which are sites with affinity for modification and restriction activities. Unmodified DNA undergoes extensive breakdown very quickly, within a few minutes after its penetration into a restrictive host (52). Experiments allowing a measurement of the frequency of the occurrence of modification and restriction have been described by Lederberg (53), and this is characteristic for each type of host bacteria.

It has been postulated that some six or seven nucleotides form a specificity site (54), and that the sites or site for modification activity is the same site as that responsible for the restriction process. Restriction of DNA is independent of DNA replication (51), nor does DNA need to undergo replication to accept modification (55). Modification is thought to involve enzymatic methylation of bases at the specificity sites, and it has been shown to require methionine as a methyl donor, presumably via S-adenosyl-methionine (SAM) (57). Restriction has also been shown to require SAM for activity of the endonuclease (57).

Several workers have endeavored to relieve modification and restriction in bacteriophage, by different means. Molholt and Fraser (58) found that E. coli, when converted to spheroplasts, would accept non-specifically modified DNA of T_2 and T_4 phages. Lederberg (59) found that heating of the restrictive bacteria in low salts medium considerably weakened restriction. Holloway (60) grew the indicator bacteria at temperatures higher than normal and found that, when returned to lower growth temperatures, P. aeruginosa would accept modified DNA and that this characteristic was retained for up to 60-70 generations before the normal host-controlled relationships were resumed. It has also been reported that modification and restriction in E. coli can be inhibited by co-infecting with another phage (61).

Lederberg (62) and Colson et al. (63) have shown the loci of the genes for modification and restriction to be linked to the threonine markers in E. coli, at about four minutes counterclockwise to the leucine marker. This work substantiated that of Boyer (64).

CHAPTER II

MATERIALS AND METHODS

Organisms

Pseudomonas aeruginosa, strain 1 (PA-1), originally obtained from B. W. Holloway of the University of Melbourne, was the source of all mutants used in this study.

Mutants PA-1-623 through PA-1-678 were isolated by Cowen (1), using nitrosoguanidine-supplemented plates (20 µg per ml) and a direct plating system.

Mutant strains A, C, D, E, F, 19, 20, and 23 were also obtained from Cowen (1). These had been isolated by treatment of PA-1 with nitrosoguanidine followed by penicillin selection (1).

Mutants PA-1-27, PA-1-55, and PA-1-64 were isolated in this laboratory by James Cowley after treatment of the wild type cells with ethyl methane sulfonate.

Mutants PA-1-503 and PA-1-506 were obtained from Green (65) and had been isolated by nitrosoguanidine treatment. Mutants PA-1-92 through PA-1-97 were also obtained from Green but had been isolated via prolonged treatment with ethyl methane sulfonate (66).

Bacteriophages used for testing mutants for phage susceptibility were obtained from R. Green. These include all phages labelled as Pϕ phage, all of which were isolated using a wild type *P. aeruginosa*

as the host. A transducing phage (F116) obtained from Holloway was also used for testing, as well as a number of phage mutants which were independently isolated during the course of the present study.

Cultivation Media

The M-9 medium described by Roberts et al. (67) was used as the base for the liquid medium employed for growth experiments. This medium contained (g per liter): NH_4Cl, 1.0; $Na_2HPO_4 \cdot 7H_2O$, 11.3; KH_2PO_4, 3.0; NaCl, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2; and distilled water to volume.

In later experiments "Pseudomonas minimal medium" (68) was used instead of M-9 salts. The two media gave comparable results. This medium contained (g per liter): $Na_2HPO_4 \cdot 7H_2O$, 8.2; KH_2PO_4, 2.7; $MgSO_4 \cdot 7H_2O$, 0.4; NH_4Cl, 1.0; $FeSO_4$ (0.1 per cent solution), 0.5 ml; distilled water to volume; the pH was 7.0 before autoclaving.

Where a solid medium was desired, agar was added to a concentration of two per cent unless otherwise stated. All supplements were sterilized separately and added to the desired concentrations as described below.

For the majority of phage studies, Pseudomonas phage medium was used for propagation of phage (22). This medium was composed of (g per liter): nutrient broth, 8.0; yeast extract, 5.0; NaCl, 5.0; distilled water to volume. The concentration of agar used was 11.0 g for the lower layer and 6.5 g for the upper layer.

In some experiments involving phage, Pseudomonas phage lactate agar (69) was used instead of the above medium. This medium consisted of (g per liter): NaCl, 5.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)H_2PO_4$, 1.0;

K_2HOP_4, 1.0; sodium lactate (60 per cent syrup), 2.0 ml; distilled water to volume. Bottom layer agar contained 11.0 g agar; top layer agar contained 6.5 g agar.

Carbon sources (sugars) were prepared at a concentration of ten per cent (w/v) and autoclaved separately; the appropriate amount of this solution was then added to give the desired final concentration in the medium. All experiments used 0.5 per cent concentration of the carbon source unless otherwise noted.

Media For Identification Of Nutritional Requirements

Yeast extract (Difco) and casamino acids (Difco, acid hydrolyzed, vitamin-free) were prepared and autoclaved at concentrations of two per cent (w/v). Each was then used in the volume of 0.05 ml of supplement to a tube containing a volume of 6.0 ml of glucose minimal medium (M-9) prior to inoculation with cells.

A vitamin mixture, containing the components listed below, was prepared and diluted 1/200 for use. A 0.05 ml volume of this diluted mixture was used to supplement 6.0 ml of M-9 salts, as with the other supplements.

Composition of vitamin mixture (amounts per 100 ml):

2.2 µg biotin
40 µg calcium pantothenate
0.2 µg folic acid
40 µg niacin
20 µg p-aminobenzoic acid
40 µg pyridoxine HCl
20 µg riboflavine
40 µg thiamine HCl

The final pH of the mixture was adjusted to 7.3; the final volume before dilution was 100 ml.

Vitamin B_{12} was also employed alone (without the presence of other vitamins). This was used at a final concentration of 5 µg per liter, and 0.05 ml was added to a volume of 6.0 ml of glucose minimal medium in test tubes.

Amino acids were prepared as solutions in deionized water at a concentration of 0.2 per cent (w/v). Each solution was sterilized by filtration through a Millipore filter (0.45 µm pore size). A 0.05 ml aliquot of this solution was used to supplement 6.0 ml of glucose minimal medium in test tubes, making the final concentration of the amino acid in the test tube 100 µg per tube, or 16.6 µg per ml.

Utilization of Precursors of Methionine

The bacterial biosynthetic pathway for the synthesis of methionine involves four intermediates of primary importance: homoserine, O-succinylhomoserine, cystathionine, and homocysteine. A procedure has been described for the synthesis of O-succinylhomoserine (70) but this compound is not yet commercially available; therefore it could not be used in growth experiments. DL-homoserine, DL allo-cystathionine, and DL-homocysteine thiolactone hydrochloride (Mann Research Laboratories, New York, N. Y.) were utilized in growth experiments as described below.

The ability to utilize the available methionine biosynthetic intermediates, or precursors, was measured by two methods, i.e., by a direct method using plates and also by measuring growth of cells in liquid medium.

For the direct plate method, 0.1 ml of a cell suspension washed from a fresh nutrient agar slant with sterile 0.85 per cent saline was

spread onto a plate of glucose minimal agar and several crystals of methionine and its precursors were separately sprinkled onto the surface of the agar in localized, marked areas. After incubation at 37° C for 24 to 72 hours, mutants were scored on the basis of presence or absence of growth. This method did not prove satisfactory as it was difficult to distinguish areas of growth from cloudiness due to the intermediate itself.

For a more accurate check, 0.2 per cent solutions of the above-mentioned intermediates were prepared, sterilized by filtration, and used as supplements to 6.0 ml of glucose minimal medium in test tubes as had been done with the amino acids previously. A 0.1 ml volume of each intermediate was used per tube, and tubes were inoculated with 0.1 ml of a cell suspension washed from a fresh nutrient agar slant with 0.85 per cent saline. Tubes were placed on a shaker at 37° C and optical density was determined periodically. This method proved to be much more effective than using plates.

Carbon Sources

Several different carbon sources were tested for their ability to support growth of the mutants. All sugars were autoclaved as ten per cent solutions (w/v) and added to liquid medium to yield a final concentration of 0.5 per cent, unless otherwise stated. Carbon sources treated in this manner included glucose, glycerol, mannitol, fructose, mannose, gluconate (K^+ salt), sorbitol, ribose, and galactose.

Other carbon sources used included histidine, pyruvate (K^+ salt), succinate (disodium salt), glutamic acid, and aspartic acid. Histidine was added at a concentration of 0.5 per cent to Pseudomonas minimal

medium and the pH of the solution adjusted to 7.0 before sterilizing by the use of a Millipore filter rather than by autoclaving. Pyruvate, succinate, glutamate, and aspartate were prepared as ten per cent solutions, sterilized by filtration, and added to a final concentration of 0.5 per cent to test tubes containing 6.0 ml of Pseudomonas minimal medium.

Glucose was employed as the carbon and energy source in growth experiments in which other supplements to the medium were used, unless otherwise noted.

Measurement of Growth

All liquid cultures were aerated by shaking on a reciprocal shaker using a total volume of 6.1 to 6.3 ml in 18 x 150 mm culture tubes with cotton plugs. The only exceptions to this were cultures for reversion experiments using ethyl methane sulfonate, for which 250 ml Erlenmeyer flasks were used, containing 50 ml of culture, and cells for enzyme experiments, which used 1000 ml flasks containing 100 ml of medium. Aeration was the same as before. Unless otherwise stated, growth temperature was 37° C.

Cell suspensions were read against appropriate blanks at 540 nm on a Coleman Junior Spectrophotometer. Readings were made at frequent intervals until the stationary growth phase had been reached. The time required to reach this stage varied with the strain and the cultural conditions.

Measurement of Glucose

Glucose remaining in the supernatant after cell growth had occurred

in the medium was measured in some experiments in order to determine the amount which had been utilized. This was done according to the Glucostat method of the Worthington Biochemical Corporation. One ml of sample containing 0.05 to 0.3 mg of glucose was added to 9.0 ml of prepared Glucostat reagent and allowed to stand for exactly ten minutes at room temperature. The reaction was then stopped by the addition of one drop of 4M HCl. The tubes were allowed to stand for at least five minutes at room temperature before being read on a Coleman Junior Spectrophotometer at a wave length of 400 nm.

Preparation of Phage Plate Stocks

Phage suspensions used for screening and testing of cultures for sensitivity to phage and also for attempted transductions were prepared according to a modification of the method described by Swanstrom and Adams (71). One drop of log phase cells and 0.1 ml of a phage suspension containing approximately 5×10^6 plaque-forming units (PFU) per ml were combined in 2.5 ml of soft Pseudomonas phage agar (top layer) and poured over the surface of a plate containing 30 ml of bottom-layer Pseudomonas phage agar. After 12 hours of incubation at 37° C, the plates were soaked with 5.0 ml of Pseudomonas phage broth for 30 minutes. The broth was then pipetted from the plates and the cells removed by centrifugation at top speed in an International clinical centrifuge for 20 minutes. The supernatant fluid was then filtered through a Millipore filter with a pore diameter of 0.45 μm. This method yielded plate stocks with titers of 10^{10} to 10^{12} PFU per ml.

Phage Titrations

Phage suspensions were titered by making dilutions of 10^{-2}, 10^{-4}, 10^{-6}, 10^{-7}, and 10^{-8} in Pseudomonas phage broth (22). Duplicate 0.1 ml samples from each dilution were plated according to the soft agar overlay method described above, using 2.5 ml of soft top layer agar and 30 ml of bottom-layer agar in plates. Pseudomonas phage agar (22) was used for the platings in most cases, but in some cases Pseudomonas phage lactate agar (69) was used in order to give more easily visualized plaques when plating the phage onto wild type cells (PA-1). Incubations were carried out at 37° C for 18-24 hours.

Tests for Susceptibility to Phage

To check for susceptibility to various phages, the cells to be checked were grown in nutrient broth for approximately 6-8 hours. One drop of this cell suspension was added to a tube containing 2.5 ml of melted, pre-cooled soft Pseudomonas phage agar, mixed, and poured onto a plate containing 30 ml of bottom-layer Pseudomonas phage agar. After allowing the top layer to solidify, suspensions of various phages to be tested were spotted from a pipette onto the top of the agar in localized, marked areas. Several phages could be spotted onto one plate by this method. After incubation overnight, plates were inspected for areas of lysis. Titers of phages spotted in this manner ranged from 10^6 to 10^{10} PFU per ml.

Transduction

Fresh nutrient agar slants of the desired organisms were prepared and incubated overnight prior to use. Each slant was flooded with 2.0 ml of 0.85 per cent saline and 0.1 ml of this cell suspension was spread over the surface of the appropriate solid medium. One drop of each phage suspension was placed in a localized, marked area on the plage and allowed to dry prior to inverting and incubating the plates at 37° C. Plates were observed for transductants after 24 to 162 hours of incubation.

CHAPTER III

EXPERIMENTAL RESULTS

Nutritional Studies

Previous Knowledge

In light of what was previously known about the growth requirements of PA-1-623 through PA-1-678, experiments were undertaken to verify the previous results and to identify the specific requirements of these mutants.

From previous data obtained by Cowen (1) it was known that these organisms, which were selected for inability to grow on glycerol, had also lost their ability to utilize glucose in minimal medium. Furthermore, the addition of 0.02 per cent yeast extract was sufficient to allow the organisms to grow on glucose again but not on glycerol. These mutants had also lost the ability to grow on sorbitol and on mannitol even with yeast extract present. They were able to grow with added yeast extract on fructose.

PA-1 was also found unable to grow on sorbitol; therefore, the loss of ability to grow on both glycerol and on mannitol was then considered to be a characteristic of these mutants. All mutants in this group (PA-1-623 through PA-1-678) appeared to possess identical growth characteristics as far as had been tested. Enzyme studies on one mutant of this group showed a normal level of glycerol kinase. However,

this mutant was shown to have no detectible α-glycerophosphate dehydrogenase activity. It was further shown to have a basal level of triose phosphate isomerase significantly lower than the enzyme activity observed for the wild type.

Two of these mutants had been shown not to be lysed by Pϕ-25 or by twelve other strains of phage tested. Lack of susceptibility to those phages tested was attributed to possible aberration of the cell wall structure which could be related to the use of glycerol as a necessary biosynthetic component. Rhamnose synthesis was suggested (1) as a possibility since glycerol has been shown to be a precursor of rhamnose. Preliminary data suggested that there was no adsorption of phage to these mutants.

The requirement for yeast extract by these mutants for growth on glucose suggested that possibly a vitamin, cofactor, or amino acid was the actual requirement.

Vitamins

To determine whether or not a vitamin was required, a solution of several vitamins was prepared and used in growth experiments with glucose as the carbon source. Also, vitamin B_{12} was tested alone. These experiments showed that the requirement was not for a vitamin, since no growth could be achieved in their presence.

Casamino Acids

Casamino acids, acid hydrolyzed and vitamin-free (Difco), were tested as a possible source of the required growth factor. This supplement was used both alone and with added tryptophan, serine,

threonine, alanine, and proline. Casamino acids alone supported growth, so it was concluded that none of the added amino acids were necessary for growth of the organisms.

Amino Acid Families

Amino acids were subdivided into five families for testing before using each alone, as suggested by Davis (72). Amino acid mixtures were composed of equal amounts of each amino acid listed, with the exception that the concentrations of cysteine and threonine were twice that of any other amino acid. The amount of supplement added to 6.0 ml of M-9 medium was such that 100 µg of each amino acid in the family was added, the only exceptions being that 200 µg of cysteine and threonine were used (concentrations are expressed as weight per 6.0 ml of medium).

Composition of amino acid families:

1. lysine, arginine, methionine, and cysteine
2. leucine, isoleucine, and valine
3. phenylalanine, tyrosine, and tryptophan
4. histidine, glutamic acid, proline, aspartic acid, and threonine
5. alanine, glycine, serine, and hydroxyproline

Amino acid families were used alone and in all possible combinations, ranging from one to all five families present in one tube. Only family 1 was found to be required for growth. These data are shown in Table I for one of the mutants, strain PA-1-623. All mutants were tested and all responded to the various supplements with very similar amounts of growth.

TABLE I

Growth of *Pseudomonas aeruginosa*, Strain 623, in Glucose Minimal Medium Supplemented With Amino Acid Families

Additions to medium (Amino acid family)	Optical Density
Family 1	0.125
Family 2	0.061
Family 3	0.052
Family 4	0.048
Family 5	0.054
Families 1 and 2	0.135
Families 1 and 3	0.129
Families 1 and 4	0.161
Families 1 and 5	0.131
Families 2 and 3	0.063
Families 2 and 4	0.068
Families 2 and 5	0.063
Families 3 and 4	0.061
Families 3 and 5	0.068
Families 4 and 5	0.071
Families 1, 2, and 3	0.132
Families 1, 2, and 4	0.143
Families 1, 2, and 5	0.128
Families 1, 3, and 4	0.141
Families 1, 3, and 5	0.125

TABLE I (continued)

Families 1, 4, and 5	0.138
Families 2, 3, and 4	0.059
Families 2, 3, and 5	0.064
Families 2, 4, and 5	0.061
Families 3, 4, and 5	0.053
Families 1, 2, 3, and 4	0.140
Families 1, 2, 3, and 5	0.129
Families 1, 2, 4, and 5	0.135
Families 1, 3, 4, and 5	0.128
Families 2, 3, 4, and 5	0.055
Families 1, 2, 3, 4, and 5	0.125

Optical densities reported in this experiment were read after 24 hours of incubation at 37°C on a shaker. It was later found that more growth could be attained using a longer incubation time, but only those samples containing Family 1 of amino acids continued to grow; the others did not.

Individual Amino Acids

After screening with amino acid families, and the discovery that amino acid family 1 contained the factor required for growth of these organisms, each individual amino acid in that family was tested. One-tenth ml of a 0.2 per cent solution of the amino acid was used with 6.0 ml of glucose minimal medium. Each amino acid from family 1 was tested separately, and methionine was found to be the requirement for growth. Cysteine would not replace methionine. With the addition of methionine to glucose minimal medium, growth to an optical density of approximately 0.7 was obtained.

The addition of family 2 of amino acids to the methionine-supplemented growth medium enhanced growth slightly. A positive effect of 0.2 to 0.3 optical density units was obtained with all but three of this series of mutants. The remaining three mutants were found to be enhanced similarly by the addition of family 4 of amino acids. These data are shown in Table II.

Carbon Sources

Utilization of Carbon Sources for Growth

Since methionine had been found to be required for growth on glucose, a study was made to determine which carbon sources could be used by mutants PA-1-623 through PA-1-678 when methionine was added to the medium. Of ten carbon sources tested, the wild type parent (PA-1) was able to grow on six, i.e., glucose, glycerol, histidine, mannitol, gluconate, and fructose, with or without the addition of methionine. The mutants were able to utilize only two of these, glucose and fructose,

TABLE II

Effect of Addition of Amino Acid Family 2 On Growth of Mutants in Glucose Minimal Salts Medium + Methionine

Mutant	O.D. without Family 2	O. D. with Family 2	Net Effect
PA-1-623	0.699	0.817	0.118
PA-1-624	0.638	0.776	0.138
PA-1-625	0.658	0.770	0.112
PA-1-626	0.638	0.886	0.248
PA-1-627	0.658	0.739	0.081
PA-1-628	0.638	0.739	0.101
PA-1-629	0.638	0.739	0.101
PA-1-630	0.620	0.770	0.150
PA-1-631	0.629	0.763	0.134
PA-1-632	0.688	0.770	0.082
PA-1-634	0.624	0.831	0.207
PA-1-635	0.653	0.789	0.136
PA-1-636	0.634	0.745	0.111
PA-1-637	0.406	0.757	0.351
PA-1-638	0.648	0.751	0.103
PA-1-639	0.453	0.776	0.323
PA-1-640	0.653	0.796	0.146
PA-1-641	0.629	0.727	0.098
PA-1-642	0.602	0.789	0.187
PA-1-643	0.643	0.763	0.120
PA-1-644	0.620	0.763	0.143

TABLE II (continued)

PA-1-645	0.638	0.782	0.144
PA-1-646	0.643	0.782	0.139
PA-1-647	0.658	0.776	0.118
PA-1-648	0.624	0.770	0.146
PA-1-649	0.565	0.757	0.192
PA-1-650	0.553	0.721	0.168
PA-1-651	0.658	0.810	0.152
PA-1-652	0.620	0.491	-0.129
PA-1-653	0.629	0.770	0.141
PA-1-654	0.648	0.615	-0.035
PA-1-655	0.598	0.745	0.147
PA-1-656	0.478	0.776	0.298
PA-1-657	0.634	0.776	0.142
PA-1-659	0.565	0.763	0.198
PA-1-660	0.658	0.745	0.087
PA-1-661	0.495	0.727	0.232
PA-1-662	0.634	0.739	0.105
PA-1-663	0.634	0.757	0.123
PA-1-664	0.602	0.694	0.092
PA-1-665	0.629	0.751	0.122
PA-1-666	0.653	0.770	0.117
PA-1-667	0.638	0.776	0.138
PA-1-669	0.620	0.796	0.176
PA-1-670	0.585	0.727	0.142
PA-1-672	0.585	0.710	0.125

TABLE II (continued)

PA-1-674	0.594	0.745	0.151
PA-1-675	0.545	0.699	0.154
PA-1-676	0.620	0.721	0.101
PA-1-677	0.638	0.634	-0.004
PA-1-678	0.553	0.658	0.105

All optical density readings reported were taken at 30 hours.

even with added methionine.

Other mutants which had been isolated as methionine auxotrophs were then tested on the carbon sources which could not be utilized by the PA-1-623 through PA-1-678 mutant group to determine whether these changes in carbon source utilization were a common accompaniment to the requirement for methionine. Mutants PA-1-92 through PA-1-97 were identical to mutants PA-1-623 through PA-1-678 in their carbon source utilization but, with the addition of methionine, mutants PA-1-27, PA-1-503, and PA-1-506 were able to grow on all the carbon sources which could not be used by mutants of the PA-1-623 and PA-1-92 series.

All these data are shown in Table III. Optical density values are given for one mutant of each group since all values were comparable. Each mutant was tested individually on all carbon sources.

Rate of Growth of Mutants

The rate of growth of mutants of the PA-1-623 and PA-1-92 series was approximately half that of the wild type on glucose minimal medium supplemented with methionine. None of the other supplements tested had increased the rate of growth. It seemed possible that glucose might be used with very low efficiency by these mutants and an experiment was designed to determine whether glucose remained after growth had ceased and whether a higher initial concentration of glucose would increase its utilization.

Duplicate samples of media containing two different concentrations of glucose were taken before inoculation and after stationary growth had been attained and were checked by the Glucostat assay. Results indicated that excess glucose was present in the medium and its

TABLE III

Utilization of Carbon Sources by
Wild Type and Mutants

Carbon Source	PA-1 (wild type)	PA-1-623 series	PA-1-92 series	PA-1-27 series	PA-1-503 series
Glucose	0.959	0.585	0.683	0.846	0.969
Glycerol	0.745	0.045	0.016	0.417	0.465
Histidine	0.699	0.027	0.039	0.688	0.921
Mannitol	0.751	0.041	0.036	0.688	0.949
Gluconate	0.450	0.022	0.035	0.174	0.335
Fructose	1.00	0.643			
Mannose	0.012	0.097			
Ribose	0.045	0.085			
Galactose	0.112	0.172			
Sorbitol	0.034	0.055			

Numbers represent optical density readings taken when cultures had reached stationary phase. Small numbers (indicating no growth) were readings obtained at 72 hours or longer.

utilization was not significantly greater at higher concentrations. These data are shown in Table IV.

Another possible explanation for the slower rate of growth of these mutants might be a defect in energy metabolism. Their inability to use histidine as a carbon source suggested a possible problem related to the Krebs cycle, since histidine is converted to glutamate which enters the Krebs cycle for further degradation or conversion to other metabolites. Therefore, it was thought that a possible block in the Krebs cycle could exist, which could be partially or totally responsible for the slow growth rate of these mutants. Accordingly, they were tested for ability to grow on several carbon sources closely related to, or included in, the Krebs cycle: glutamate, aspartate, pyruvate, and succinate.

The results of these experiments are shown in Table V. It was found that both groups of mutants could grow on either succinate or pyruvate in the presence of methionine. Therefore, no complete block in the Krebs cycle could be present. However, neither group could utilize glutamic acid and therefore it is apparent that their inability to grow on histidine is actually due to inability to utilize glutamate. With aspartic acid, growth occurred very slowly over a period of 72 hours. Therefore, these organisms can utilize aspartate, although very slowly.

Crossfeeding Studies

Several methods were tried to obtain successful crossfeeding between the different mutants.

TABLE IV

Utilization of Glucose by PA-1-623

Tube No.	Glucose concentration before inoculation	Glucose concentration after growth (in supernatant)
1	4.9 mg/ml	2.4 mg/ml
2	4.6 mg/ml	1.6 mg/ml
3	13.0 mg/ml	9.5 mg/ml
4	12.0 mg/ml	9.0 mg/ml

Cultures were grown in glucose minimal medium supplemented with methionine and amino acid family 2. Cultures were grown for 35 hours and had reached optical density readings of 0.8 to 0.9.

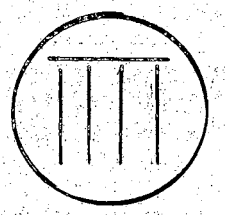
Concentrations of glucose were measured by use of the Glucostat assay.

TABLE V

Growth of PA-1-631 and PA-1-92 on Carbon Sources Related To The Krebs Cycle (Optical Density Readings)

Carbon Source and Time	Organism PA-1	PA-1-631 - Meth	PA-1-631 +Meth	PA-1-92 -Meth	PA-1-92 +Meth
Glutamate					
24 hr	0.643	0.039	0.036	0.082	0.062
48 hr	1.097	0.068	0.043	0.129	0.086
72 hr	-	0.068	0.043	0.129	0.086
Aspartate					
24 hr	0.569	0.046	0.076	0.087	0.098
48 hr	0.638	0.048	0.114	0.057	0.229
72 hr	-	0.048	0.174	0.057	0.357
Succinate					
24 hr	0.472	0.054	0.611	0.052	0.602
48 hr	0.412	0.052	0.491	0.049	0.488
72 hr	-	0.052	0.491	0.049	0.488
Pyruvate					
24 hr	0.602	0.056	0.469	0.059	0.520
48 hr	0.538	0.056	0.573	0.058	0.638
72 hr	-	0.056	0.573	0.058	0.638

In the first method, cells were grown on nutrient agar slants, and a small inoculum of one mutant was streaked vertically along plates of glucose (0.5 per cent) minimal agar, supplemented with 0.33 µg/ml methionine to allow cells to begin growth, streaking near the edge of the plates. The other mutants to be tested were streaked horizontally along the plate, streaking just up to the line of the first streak, with care being taken not to touch the inoculum of the other mutants. All mutants were tested in all possible combinations, following the pattern shown below:



According to the theory of crossfeeding, each mutant may build up high levels of the intermediate before its own block, and may excrete this intermediate into the medium. The intermediate diffuses slightly through the agar and is then available to the other mutants for growth. Only those mutants which are blocked in a step prior to the intermediate are able to use it and grow. Any mutant that can be fed by another mutant will have a block in its pathway before that of the mutant which is able to feed it. Crossfeeding data give an indication of the order of the steps in a pathway. This method gave poor results and no

crossfeeding was obtained.

Another method consisted of pouring fifteen ml of agar (the same as used above, supplemented with 0.33 µg/ml methionine) into a Petri plate, allowing it to harden, and spreading 0.1 ml of a saline suspension of one mutant on the surface of the plate. After it had been allowed to dry, other mutants were streaked in designated areas on top of this. This method also gave no conclusive results.

Successful crossfeeding was obtained by following a slight modification of the method of D. H. Calhoun (personal communication). Fifteen ml of melted, precooled glucose minimal agar (1.5 per cent agar), supplemented with 0.1 µg/ml of methionine, were inoculated with 0.4 ml of a washed cell suspension (0.85 per cent NaCl) from an overnight nutrient agar slant. This was poured into a Petri plate, allowed to solidify, and spotted with one drop of a similar suspension of each of the other mutants. Plates were kept level until the drops had dried to avoid spreading of the drops which had been spotted on top. Plates were observed for growth after two to four days when growth could be seen in the area where the cells had been spotted, indicating growth of one of the strains had occurred due to the feeding of nutrients from one strain to the other. To determine which of the strains was growing, the plates were read, then the area of growth was scraped away, being careful not to tear the surface of the agar. The plates were then read again. If the cells in the agar had been growing, nearly all of the apparent growth would still remain, indicating that the cells on the bottom (seeded into the agar) had been fed by those spotted onto the top of them. If the topmost cells had grown because of being fed by the cells in the agar, then nearly all visible growth would have been

removed from the area. Best observations were made when the cells that were fed were located in the agar, but all crossfeeding experiments were done with each mutant seeded into the agar as well as spotted onto the top of the agar.

Data from these experiments are given in Table VI. According to these data, PA-1-92 and PA-1-623 were fed by PA-1-27, PA-1-503, and PA-1-506, indicating that the latter three mutants are blocked at a point in the pathway subsequent to that at which the former mutants are blocked.

Attempted Isolation of Revertants

In all experiments which had been carried out with mutants PA-1-623 through PA-1-678, no reversion had been observed. In view of the multiple differences between these mutants and the wild type, it appeared possible that these were deletion mutants. If so, it should not be possible to obtain reversion even by treatment with mutagens. Therefore, repeated attempts were made to isolate revertants using two different mutagens which are highly effective with P. aeruginosa: nitrosoguanidine and ethyl methane sulfonate.

Glucose minimal agar plates were prepared containing, in addition to all normal components, 5.0 µg per ml of nitrosoguanidine. Plates were poured with 30 ml of agar per plate. The nitrosoguanidine was sterilized separately by filtration and added to the agar at the proper concentration just before pouring the plates. Each plate was spread, when solidified, with 0.1 ml of a stationary phase culture grown in M-9 glucose minimal medium supplemented with 33.3 µg of methionine per ml (0.1 ml of a 0.2 per cent solution of methionine in 6.0 ml of

TABLE VI

Crossfeeding Experiments

	Cells in Agar				
Fed by	PA-1-27	PA-1-92	PA-1-623	PA-1-503	PA-1-506
PA-1-27	-	+	+	-	-
PA-1-92	-	-	-	-	-
PA-1-623	-	-	-	-	-
PA-1-503	-	+	+	-	-
PA-1-506	-	+	+	-	-

medium). Plates were incubated for one week, and were checked for growth daily.

For ethyl methane sulfonate experiments, a slight modification of the procedure used by Nečásek et al. (66) was employed. A nutrient broth culture of PA-1-623 was grown to a concentration of 3 x 10^9 cells per ml and diluted 1/3 with 0.067 M potassium phosphate buffer, pH 7.2, using enough to have a final volume of 20 ml. This cell suspension was transferred to sterile centrifuge tubes and centrifuged at 3,000 x g in a Sorvall RC-2B refrigerated centrifuge at 4°C for 15 minutes, then resuspended in the same volume of buffer. This was repeated four times. The 20 ml of culture was then transferred to a sterile 50 ml Erlenmeyer flask and 0.26 ml of ethyl methane sulfonate was added and shaken slightly to dissolve, and the flask was allowed to remain at room temperature without shaking. Samples of 5.0 ml each were taken at time intervals of 4, 6, 8, and 18 hours (in some experiments 4, 8, 12, 14, and/or 18 hours were used) and the cells were centrifuged as before. The supernatant containing the EMS was discarded and the remaining cells were resuspended in 2.0 ml of 0.85 per cent NaCl. One-tenth ml samples of this final suspension were spread onto glucose minimal agar plates. At least ten plates were made from each timed sample. Plates were kept under observation for one week to ten days.

Neither the nitrosoguanidine method nor the ethyl methane sulfonate method proved successful in producing revertants, even after repeated attempts. This finding is consistant with the idea that these mutants are deletion mutants.

Utilization of Intermediates

All mutants were tested for ability to utilize each of the available intermediates in the methionine biosynthetic pathway, to determine at which point the blockage had occurred. Figure 3 shows the pathway and the apparent blockage points. All mutants of the PA-1-623 through PA-1-678 series appeared to be blocked in the same enzymatic step. Mutants PA-1-92 through PA-1-97, PA-1-27, and PA-1-64 also seemed to be blocked in the same step, the conversion of homoserine to cystathionine. Mutants PA-1-503 and PA-1-506 appeared to be blocked in the conversion of cystathionine into homocysteine, the next sequential step in the pathway.

Studies With Phage

Phage Adsorption Studies

Cowen (1) had found that mutants of the PA-1-623 through PA-1-678 series were not lysed by any of 13 phages capable of lysing the wild type parent. Green (65) had found that PA-1-27, PA-1-503, and PA-1-506 were susceptible to phages F116 and Pϕ-25, but mutants PA-1-64 and those of the PA-1-92 through PA-1-97 series were not susceptible to either of these phages. Since loss of susceptibility to phage could result either from alteration in the cell wall to prevent adsorption or from alteration or loss of a metabolic function of the cell which is necessary for phage replication, experiments were carried out to determine whether the phage were adsorbed onto these mutants.

Pϕ-25, grown on PA-1, was used at a concentration of approximately 1.4×10^8 PFU per ml in adsorption experiments. The concentration of

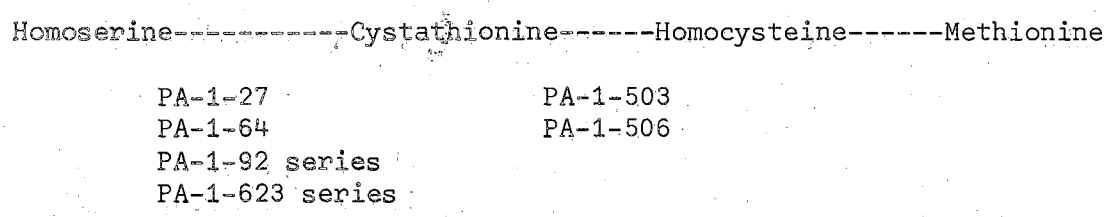


Figure 3. Apparent Enzymatic Defects of Mutants Based on Ability to Utilize Methionine Precursors

cells was varied in different experiments. The ratio of phage to cells was between 1.0 and 2.0. PA-1-623 was grown for 6 hours in nutrient broth and diluted to an optical density of 0.078. (This insures that the cells are in log phase). Five ml of culture were added to a sterile 50-ml Erlenmeyer flask, and 3.0 ml of phage suspension added to another 50-ml flask. Both flasks were equilibrated to 38°C in a gently shaking water bath. At time zero (T_o), 3.0 ml of cell suspension were added to the flask containing the phage. From the remaining 2.0 ml of cell suspension, 0.1 ml samples were taken and diluted for plating on nutrient agar plates for a total cell count. For the initial phage titer, a sample of the original phage suspension was diluted in Pseudomonas phage broth, and the last dilution tube placed in an ice bath. Samples of 0.1 ml were taken from the experimental flask after 5, 10, 15, 20, 30, 45, 60, and 90 minutes and each was put into 9.9 ml of Pseudomonas phage broth. After mixing, a five ml sample of this suspension was centrifuged for 6 minutes at top speed in an International clinical centrifuge to remove cells. The supernatant was diluted 1/100 into pre-cooled phage broth and stored in ice until it was plated. Duplicate 0.1 ml samples were plated onto Pseudomonas phage lactate agar, using the soft agar overlay method. PA-1 was used as the host. Plaques were counted after overnight incubation at 37°C.

In order to allow sampling at more frequent time intervals, cells were removed by lysis with one drop of chloroform in the first dilution tube and the centrifugation step was omitted. A phage control was run with and without chloroform. The effect of chloroform on the viability of the phage was checked by incubating a sample of phage with chloroform and sampling at timed intervals. Table VII presents data obtained

TABLE VII

Effect of Chloroform on Viability of Phage Pϕ-25

Sample minutes	Plaques per plate	Titer (PFU/ml)
0	103, 82, 93 73, 97, 99	9.1×10^7
2	102, 90, 100, 86	9.5×10^7
4	80, 75, 71, 73	7.5×10^7
6	68, 85, 78, 76	7.7×10^7
8	58, 51, 57, 47	5.3×10^7
10	56, 41, 35, 46	4.5×10^7

Phage Pϕ-25 was incubated at 38°C for 10 minutes in Pseudomonas phage broth plus 2.5×10^{-3} M $CaCl_2$. After incubation, a 0.1 ml sample was taken and diluted to 10^{-5}, the first dilution tube containing 2 drops of $CHCl_3$ in 9.9 ml of Pseudomonas phage broth. Time in the table refers to the interval between the addition of the phage sample to the first dilution tube and its subsequent further dilution to 10^{-5}. Phage was not exposed to any cells for adsorption. Titering was done using PA-1 as the host. The initial phage titer on PA-1 was 1.08×10^8 PFU/ml.

in this manner. Table VIII compares lysis by chloroform and centrifugation as effective means of removing cells. Both methods are effective but the data shown in Table VII indicate that chloroform should not be used if the phage must be left in contact with it for more than a few minutes.

The effect of incubation at 38°C on the viability of the phage was tested by incubating a 2-ml sample of phage in a separate flask at the same temperature simultaneously with the experiments for adsorption studies.

The effect of $CaCl_2$ on phage adsorption was tested by incubating one flask containing 2.5×10^{-3} M $CaCl_2 \cdot 6H_2O$ in addition to the phage and cells.

Table IX gives data for the effect of both heat and $CaCl_2$ on the adsorption of phage to the mutant cell strain PA-1-623. These data show that incubation at 38°C for the time required to perform an adsorption experiment has no significant effect on the phage titer as expressed in PFU per ml. Data also indicate that $CaCl_2$ does not aid in adsorption of this particular phage strain to the group of mutant cell strains used. Therefore, in further experiments, no $CaCl_2$ was added to the flask used for carrying out adsorption studies.

In several such experiments no adsorption of phage Pϕ-25 onto mutant PA-1-623 could be measured during time intervals of 5 through 90 minutes. Table X shows data for a typical experiment.

This phage was found to be sensitive to prolonged exposure to chloroform (Table VII) and insensitive to exposure to 38°C (Tables IX and X). Adsorption was not brought about by the addition of $CaCl_2$ at a level of 2.5×10^{-3}M (Table IX).

TABLE VIII

Phage Adsorption Experiments with PA-1-623 and Phage Pϕ-25

Original Phage titer (PFU/ml)	Time Allowed for Adsorption	Method of Cell Removal	Titer after Exposure to PA-1-623 (PFU/ml)
6.32×10^8	5 minutes	Centrifugation	5.92×10^8
1.48×10^8	10 minutes	Centrifugation	1.44×10^8
1.08×10^8	10 minutes	$CHCl_3$	1.11×10^8
1.62×10^8	15 minutes	Centrifugation	1.66×10^8
8.5×10^7	20 minutes	Centrifugation	9.0×10^7

Centrifugation for removal of cells was done at top speed in an International clinical centrifuge for 10 minutes, and 0.1 ml samples were taken from the supernatant for proper dilution and plating. $CHCL_3$ was added in the one case at a level of 2 drops in the first dilution tube. The phage were in contact with the $CHCl_3$ less than one minute. PA-1 cells were used for determining phage titer.

TABLE IX

Results of Phage Adsorption Experiment Showing Effect of Temperature and $CaCl_2$ on Adsorption of Phage

Sample	Dilution	Count	Final Titer (PFU/ml)
T_o phage	2.5×10^{-6}	187, 197, 164, 182, 172, 170	7.16×10^{8}
Phage at 38°C	1.25×10^{-6}	85, 90, 101, 81, 97, 82	7.12×10^{8}
Phage + PA-1-623 cells	1.25×10^{-6}	69, 76, 79, 74, 70, 75	5.92×10^{8}
Phage + Cells + $CaCl_2$ (2.5×10^{-3}M)	1.25×10^{-6}	80, 74, 94, 90, 60, 74	6.32×10^{8}
PA-1-623 cells	1×10^{-5}	312, 317	3.13×10^{8} cells/ml

Ratio of phage to cells was 2; time allowed for adsorption was 5 minutes in this experiment.

TABLE X

Phage Adsorption Experiment With PA-1-623
At Different Time Intervals

Sample	Titer of Phage (PFU/ml)
T_o phage, no $CHCl_3$	2.6×10^8
T_o phage + one drop $CHCl_3$ in first dilution tube	3.4×10^8
5 min. adsorption	1.9×10^8
10 min. adsorption	1.9×10^8
20 min. adsorption	2.3×10^8
30 min. adsorption	1.8×10^8
45 min. adsorption	2.1×10^8
60 min. adsorption	1.5×10^8
90 min. adsorption	2.0×10^8

Pϕ-25 phage at a concentration of 2.6×10^8 PFU/ml was mixed with PA-1-623 cells and allowed times of 5, 10, 20, 30, 45, 60, and 90 minutes to adsorb while being gently shaken in a 38°C water bath. Samples of 0.1 ml were taken at the designated time intervals and diluted appropriately before plating on Pseudomonas phage agar. PA-1 cells were used for titering phage samples.

Phage Sensitivity of Various Mutants

Since transduction studies would be very helpful in genetic analysis of methionine biosynthesis in P. aeruginosa, extensive efforts were made to obtain phage which could be used with all the mutants.

Each mutant in the PA-1-623 through PA-1-678 series was tested for sensitivity to each of 34 different known Pseudomonas phages capable of infecting and causing lysis in the wild type parent. None of these phages were found capable of infecting any member of this series, nor could they infect and cause lysis in any member of the PA-1-92 through PA-1-97 series. These same phages caused lysis of PA-1-27, PA-1-503, and PA-1-506. With a few exceptions, PA-1-55 and PA-1-64 were found to be resistant to these phages. Mutant strains A, C, D, E, F, 20, and 23 appeared sensitive so far as tested.

Seven phages (designated Group I phage) were isolated independently during the course of this study. These phages were found able to cause lysis in all members of the PA-1-623 through PA-1-678 series and in the PA-1-92 through PA-1-97 series. However, these phages were incapable of infecting any of the other cell strains used in this study or the wild type parent (PA-1). The source of these phage was attributed to either mutation of a prophage or chance contamination. Taylor (73) has reported a similar isolation which he attributed to contamination.

A second series of phages (Group II phage) isolated during this study caused lysis of PA-1 (wild type) and all mutants except those belonging to the PA-1-623 through PA-1-678 series, the PA-1-92 through PA-1-97 series, and PA-1-55 and PA-1-64. No exceptions were found in

these series of mutants. Table XI shows a summary of the phage sensitivities of various mutants used in this study.

Since no phage had been found capable of lysing both the wild type (PA-1) and mutants of the series PA-1-623 through PA-1-678 and PA-1-92 through PA-1-97, extensive efforts were made to isolate another mutant organism which would be useful in transduction studies. In order to be most useful, the desired mutant must possess wild type characteristics with respect to glycerol and methionine metabolism, yet be susceptible to Group I phage.

In attempts to obtain this desired mutant, PA-1 cells were inoculated into 6.0 ml of glycerol minimal medium containing 5.0 µg per ml of acriflavin and grown to stationary phase. The cells so obtained were washed once and resuspended in Pseudomonas minimal medium without carbon source and used as inoculum into fresh glycerol minimal medium. After reaching late log phase, one drop of this cell suspension was placed into a tube containing 2.5 ml of soft Pseudomonas phage agar along with 0.1 ml of a suspension of Pφ-6 at a concentration of approximately 10^9 PFU per ml, mixed, and poured over the surface of a bottom-layer agar plate. After incubation overnight, colonies were picked and streaked onto nutrient agar plates to remove any phage which may have remained. Then each culture was checked for susceptibility to Group I phage. One hundred and seventy-five isolates obtained by this method were checked for susceptibility to Group I phage, and none were found which were sensitive to these phages. One mutant isolated during the acriflavin exposure treatment was found to have a degree of susceptibility to Group I phage, but at markedly reduced levels. This mutant, strain 7, will be discussed later (under Modification and Restriction).

TABLE XI

Phage Sensitivities of Various Mutants

Cell Strain	Phage Group		
	stock wild type phage	Group I phage	Group II phage
PA-1-623-678	resistant	sensitive	resistant
PA-1-92-97	resistant	sensitive	resistant
PA-1-27	sensitive	resistant	sensitive
PA-1-55	resistant*	resistant	resistant
PA-1-64	resistant*	resistant	resistant
PA-1-503	sensitive	resistant	sensitive
PA-1-506	sensitive	resistant	sensitive
A		resistant	sensitive
C		resistant	sensitive
D		resistant	sensitive
E		resistant	sensitive
F		resistant	sensitive
20		resistant	sensitive
23		resistant	sensitive
PA-1	sensitive	resistant	sensitive

*PA-1-55 was found sensitive to two phages in this group;
PA-1-64 was found sensitive to three phages in this group.

Another method utilized to obtain the desired organism was as follows. PA-1 cells were inoculated along with PA-1-623 cells into the same tube of 6.0 ml of glucose minimal medium supplemented with methionine at the same concentration as before. After the cell suspension had reached stationary growth, one drop of this mixture of cells was added to 2.5 ml of soft Pseudomonas phage agar along with 0.1 ml of Pϕ-6 suspension at a concentration of approximately 1 x 10^9 PFU per ml and poured onto a bottom-layer agar plate. After growth, colonies were streaked onto nutrient agar plates to remove any remaining phage and then tested for susceptibility to Group I phage in the manner described previously. The rationale for this experiment was provided by Luria's statement that, in some instances, the modifying activity of the host bacterium is due to lysogeny for another, unrelated phage (74). Growth of the two strains together would allow opportunity for each to be lysogenized by phage carried by the other strain, if there were differences of this type between the two strains. No colonies with altered phage susceptibility were found.

Transduction

Transduction studies were performed according to the technique described by Murphy and Rosenblum (75) with a slight modification. Cells were washed from a fresh overnight nutrient agar culture with 2.0 ml of 0.85 per cent NaCl and 0.1 ml of this suspension was spread onto a minimal agar plate. One drop of phage plate stock was placed in a localized area on the surface of the plate and allowed to dry before inversion of plates. At least six different phage plate stocks could be tested on each plate.

By the use of this method, attempts were made to obtain transduction between mutants PA-1-27, PA-1-503, PA-1-506, and glycerol mutants 20, 23, and E using both Group I and Group II phage. None were found to be effective in producing transductants. Group I phage was used in transduction attempts between mutants of the PA-1-623 through PA-1-678 series with no success. The same results were obtained with the PA-1-92 through PA-1-97 series when using Group I phage.

The medium used in most transduction attempts was glucose minimal medium agar plates. In some cases, glycerol minimal agar plates were used also to observe possible co-transduction of both the methionine and glycerol markers (in experiments involving only the PA-1-623 through PA-1-678 series and the PA-1-92 through PA-1-97 series). The agar plates were used both with and without supplementation with 0.1 µg per ml of methionine.

In transduction attempts between strain 7 and PA-1-631, another method in addition to that previously described was also attempted. Phage ϕ-631 grown on strain 7 cells was added to PA-1-631 cells in test tubes containing glucose minimal medium and glycerol minimal medium and placed on a shaker in an effort to obtain growth. Any growth obtained in such manner would indicate transduction since only supposed transductants could grow in these media. However, these experiments also failed to produce any transductants.

Modification and Restriction

As described above, of 175 colonies tested only one (strain 7) was found to have at least partial susceptibility to phages of both Group I and Group II. Since this was the only bacterial strain

available which offered some promise of usefulness in transductions between the wild type and the mutants of the glycerol-negative, methionine-negative, phage-resistant type, it was investigated further.

This mutant showed a slight sensitivity to phage from Group I, but at markedly reduced levels (see Table XII). When grown on PA-1-631, Group I phage would plate on strain 7 cells at an effeciency that was only 10^{-4} to 10^{-5} times the efficiency of plating on PA-1-631. When grown on strain 7 cells, the phage would plate on PA-1-631 at only 10^{-2} to 10^{-3} times the efficiency of plating on strain 7 cells. This is typical of phage-cell systems which exhibit host-controlled modification (50).

Several methods to relieve modification and restriction have previously been reported. With E. coli, restriction of T_2 and T_4 DNA has been reversed by conversion of the non-permissive cells into spheroplasts (58). Holloway (60) has reported that in P. aeruginosa the ability of non-accepting strains to plate host-modified phage is acquired in approximately three generations of growth at 43°C; the cells can then be returned to growth at 37°C. Once acquired, this ability is retained for a period of up to 60 to 70 generations at 37°C. This method was attempted with PA-1-7; however, these cells lacked the ability to grow at 43°C, so they were grown at gradually increasing temperatures up to the maximum temperature which would allow growth. No growth could be attained above 41°C, and after growth at this temperature cells still exhibited the same degree of modification and restriction. Consequently, no transductions could be done with this organism.

TABLE XII

Modification and Restriction Studies with PA-1-7 and Group I phage (with PA-1-631 as alternate host)

Phage strain	Grown on	Titer on PA-1-7 (PFU/ml)	Titer on PA-1-631 (PFU/ml)
ϕ-631	PA-1-7	8.3×10^6	3.0×10^4
ϕ-632	PA-1-7	8.5×10^7	1.7×10^4
ϕ-631	PA-1-631	4.3×10^2	4.8×10^6
ϕ-632	PA-1-631	3.0×10^2	1.3×10^7

Enzyme Assays

Assays for the enzymes of the methionine pathway are dependent upon a method for detection of the product of the reaction, and published methods have used labeled substrates and have detected products by radioautography of chromatographed reaction mixtures. It seemed worthwhile to attempt to carry out these enzyme assays using standard chromatographic methods for detection of the products since enzyme data would confirm the positions of the enzymatic defects in the mutants.

Assays for these enzymes were attempted first with the wild type (PA-1) before applying the methods used to the mutant organisms. The following method was used for assay, based on a composite of assays of similar type reported by other workers (7, 10, 14, 76). A cell-free extract of PA-1 was produced by sonication and the protein concentration of the resulting extract was determined by the method of Sutherland et al. (77). A reaction mixture was prepared containing the following amounts of each component, given as the amount contained in one ml of reaction mixture: homoserine, 5 μ moles; cysteine, 12 μ moles; phosphate buffer, (pH 7.5) 64 μ moles; ATP, 6 μ moles; $MgCl_2$, 6 μ moles; glucose, 12 μ moles; succinate, 30 μ moles. To furnish additional cofactors which might be required, in some experiments the reaction mixture also received one of the following additions: 0.1 ml of a boiling water extract of whole cells; 0.2 μ moles of pyridoxal phosphate; 200 μ g of coenzyme A; or both pyridoxal phosphate or coenzyme A.

The reaction was initiated by addition of cell-free extract containing 2 μg protein, and incubation was carried out at 37°C in a water

bath. After one hour of incubation, the reaction tube was removed from the bath and the contents were divided into two equal portions. One portion was used for chromatography without further treatment. Protein was precipitated from the other portion either by heating in a boiling water bath for 5 minutes or by addition of 0.5 ml of 10 per cent trichloroacetic acid.

Samples of the reaction mixtures were spotted onto Whatman No. 1 paper strips, 60 cm in length, and were subjected to descending paper chromatography. Two-dimensional ascending paper chromatography on 23 x 23 cm squares of the same paper was also used in some experiments.

After the samples had been applied to the paper, the chromatograms were developed in the following solvent systems:

1. Butanol: acetate: H_2O; 2:1:1 (7).
2. 80 per cent phenol in H_2O.
3. Butanol: formate: H_2O; 77: 10: 13. Samples were first oxidized by adding 0.5 volume of formaldehyde and allowing them to sit for 24 hours at room temperature (78).
4. (a) t-Butanol: HCl: H_2O; 70: 6.7: 23.3.
   (b) Isopropanol: formate: H_2O; 70: 10: 20.
   This solvent system (70) was also used for one-dimensional chromatography, using each one separately.

Standard solutions of homoserine, homocysteine, cystathionine, methionine, and cysteine were always developed (chromatographed) simultaneously with the reaction mixtures. Development of chromatograms in isopropanol (70): formate (10): H_2O (20) produced satisfactory separations with a mixture of the standards but separation of the reaction mixtures was difficult to achieve.

As a method of detection of the various compounds, ninhydrin (0.5 per cent) in either butanol or acetone gave satisfactory results with

standards, but the reaction mixtures contained too many ninhydrin-positive compounds to allow positive identification of cystathionine. A more specific detection reagent, iodine-azide (78) did not prove satisfactory with reaction mixtures. Nitroprusside reagent (78) gave positive results for cysteine and homocysteine standards only at levels of 20 µg or more and did not prove satisfactory with reaction mixtures. It was concluded that enzyme assays are not feasible until a better assay and/or a better and more sensitive means of detection of the various compounds is found.

CHAPTER IV

DISCUSSION

The data obtained throughout this study give information concerning several defects of mutants of Pseudomonas aeruginosa and permit the formation of several conclusions and postulations about the character of these mutants and the organization of the genome of this organism.

Growth data indicate that mutants PA-1-623 through PA-1-678 are methionine auxotrophs. Since methionine cannot be replaced by cysteine, this is not a sulfur requirement. Furthermore, these mutants have also been shown to lack the ability to utilize glycerol, gluconate, histidine, and mannitol as carbon sources. The histidine defect has been shown to be due to inability to utilize glutamic acid. Phage resistance is also a characteristic of these mutants.

These mutants are thought to be the result of deletions because of their inability to revert, either spontaneously or by being induced with mutagens. This lack of reversion is usually associated only with deletion mutants. Due to the multiple defects of these organisms, it is thought that the deletion hypothesis is much more likely than for such numerous point mutations to have occurred. Since all mutants were isolated independently yet possess the same defects, it is doubtful that so many point mutations could have occurred at the same points.

Another series of mutants, PA-1-92 through PA-1-97, isolated after ethyl methane sulfonate treatment, were found to possess defects

identical to those of the PA-1-623 through PA-1-678 group. These two groups of mutants are identical in every respect, including the inability to revert. This gives further support to the thought that these are deletion mutants.

A group of phages was isolated which cause lysis in both of the above mentioned groups of mutants but which will not infect the wild type (PA-1). This group of phages is specific for these two groups of mutants.

All other mutants used in this study (PA-1-27, PA-1-64, PA-1-55, PA-1-503, PA-1-506, and the A through F series) possess reversion rates, of varying degrees. These organisms do not possess the phage properties of the above two mutant groups, nor do they lack the ability to utilize gluconate, mannitol, or histidine. Their defects are confined to either a methionine or a glycerol mutation.

Studies involving the use of the available precursors of methionine in growth experiments have yielded information concerning the apparent blocked points in methionine synthesis (see Figure 3). From the utilization of precursors, there appear to be only two loci involved in the blockage of the methionine pathway in these mutants. PA-1-27, PA-1-64, PA-1-92 through PA-1-97, and PA-1-623 through PA-1-678 all appear to lack an enzyme which converts homoserine into cystathionine, while PA-1-503 and PA-1-506 seem to lack only β-cystathionase. No mutants were found which lacked homocysteine methylase. All methionine mutants responded to homocysteine. However, since O-succinylhomoserine was not available for testing, no means of differentiating the two steps between homoserine and cystathionine could be used, and the two steps could not be distinguished by growth studies.

Crossfeeding data were obtained and used to confirm the existence of two steps between homoserine and cystathionine (see Table V). Since PA-1-27 will feed PA-1-92 and PA-1-631, they cannot all be blocked or deficient in the same enzyme. PA-1-92 and PA-1-631 must be blocked at a point earlier in the pathway than PA-1-27, since crossfeeding can occur only in this manner. It then follows that PA-1-92 and PA-1-631 must lack homoserine succinylase and that PA-1-27 must lack cystathionine-γ-synthase.

Since PA-1-503 and PA-1-506 will feed PA-1-92 and PA-1-631, yet will not feed PA-1-27, and because growth on precursors indicates that PA-1-27, PA-1-503, and PA-1-506 are not all blocked in the same enzymatic step, then PA-1-503 and PA-1-506 must lack β-cystathionase. Since these two mutants will not feed PA-1-27, it is possible that these organisms may lack both cystathionine-γ-synthase and β-cystathionase activities. This theory would explain the lack of crossfeeding with PA-1-27.

Since both these mutants revert, a deletion cannot be postulated to account for the loss of two enzyme activities. If both activities are lacking in these mutants, two explanations can be suggested for a loss of two enzymes as a result of a single site mutation. First, there might be a single peptide which is common to both enzymes, possibly combining with two different peptides to produce the two enzymes. A mutation in the common peptide could cause the loss of both activities, while a mutation in either of the other peptides would cause the loss of only one enzyme activity. A second possibility is that PA-1-503 and PA-1-506 are reading frame mutants and that the loci for cystathionine-γ-synthase and β-cystathionase are contiguous on the genome

so that both would be affected by a single mutation. The apparent revertants could then be true revertants or suppressor mutants in which a second mutation restores the proper reading frame.

A survey of the combined results of studies with precursors and those with crossfeeding, when compared to the various other defects present in the various mutants, can be used to indicate patterns of similarity. All met$^-$ mutants which cannot metabolize glycerol are deficient only in homoserine succinylase. Since both the PA-1-623 through PA-1-678 series and the PA-1-92 through PA-1-97 series are defective in both glycerol utilization and methionine biosynthesis, even though selected for only one defect, this suggests that there may be a possible linkage between these two loci. None of the other enzymes in the synthesis of methionine appear to be involved in this close proximity. Both of these mutant groups possess the same phage properties also, indicating that a locus for phage resistance may also be located near to the met 1 enzyme and the enzyme responsible for inability to utilize glycerol. All glycerol mutants which do not revert also lack the met 1 enzyme as well as possess resistance to phage.

Since all mutants which possess the defects mentioned above also are unable to utilize gluconate, mannitol, and histidine, it is possible that the loci for utilization of these compounds are also contiguous to those mentioned above. Only the mutants which cannot utilize glycerol, synthesize methionine, or be lysed by phage also possess defects in gluconate, mannitol, and histidine metabolism. Since no other mutants possess all of these defects, it is thought that the defects may all be a part of the same segment of the genome which has been deleted. Before these theories can be proven, additional mutants must

be isolated and tested for the same defects and patterns of similarity.

The enzymes of the glycerol pathway which are of interest in this study are a non-NAD-linked L-α-glycerophosphate dehydrogenase and triose phosphate isomerase. Work done by Cowen (1) has shown that the PA-1-623 through PA-1-678 series of mutants possess no L-α-glycerophosphate dehydrogenase and have a very low basal level of triose phosphate isomerase, much lower than that found in the wild type. These cells still possess glycerol kinase, however. Cowen has shown that glycerol mutants C, E, F, and 20 (mutants which are defective only in glycerol metabolism) have no common lesion. Mutants C, E, and 20 had dehydrogenase activities comparable to that of the wild type, while mutant F lacked this activity. Mutant E had low isomerase activity.

From these observations it seems possible that the isomerase locus is farther away from the methionine and phage resistance loci than is the dehydrogenase locus. Mutant E possesses a low isomerase activity and a normal dehydrogenase activity, while mutants PA-1-623 through PA-1-678 have a low isomerase activity but completely lack measurable dehydrogenase activity. Thus, one would expect the above positioning to be the most valid theory.

In attempts to use transduction for analysis of all mutants involved in this study, no phages could be obtained which would allow these studies to be performed. In attempts to isolate another mutant which would allow these studies to be done, one mutant (strain 7) was obtained which appeared to be of possible use in such experiments. However, modification and restriction prevented crosses between this strain and the mutant strains of the PA-1-92 and PA-1-623 series.

Two methionine mutants, PA-1-64, and PA-1-55, had previously been thought to be resistant to phages capable of infecting wild type cells. Two "wild type" phage were found to be able to infect these cells and cause lysis. These organisms are blocked between homoserine and cystathionine, as found by growth data. The two phages which would lyse both cultures (Pϕ-5 and Pϕ-17) were used in transduction studies between PA-1-27, and PA-1-55, and PA-1-64 to determine whether these three mutants are blocked in the same enzyme. Due to a high reversion rate in PA-1-55 and PA-1-64, no conclusive data could be obtained.

The cause of phage resistance in PA-1-623 through PA-1-678 has been determined to be due to a complete lack of adsorption of the phage to the cells. Experimental conditions utilized to measure adsorption were checked to be certain that no decrease in viability of the phage during the period of the adsorption experiments occurred. The lack of adsorption is probably due to some aberration in the structure of the cell wall, since phage receptor or attachment sites are located here. This situation could exist if the mutants involved are incapable of synthesizing some necessary intermediate required for structure of the cell wall, and may or may not be related to the inability to utilize any of the carbon sources which was reported herein.

The inability of PA-1-631 and PA-1-92 to utilize histidine as a sole carbon source has been found to be due to an inability to utilize glutamic acid (see Tables III and V). Since this compound is fed directly into the Krebs cycle, it was decided to check for possible defects in the Krebs cycle. If such were the case, the slow growth rate of both of these mutant groups (slightly less than half the rate of the wild type on glucose) could possibly be explained. Data indicate that

no such defects occur, since both organisms grew quite well on pyruvate and succinate in the presence of methionine. The organisms grew on these compounds at a rate faster than the rate at which they can utilize glucose. The growth rate on aspartic acid was very slow.

Since these mutants cannot utilize glutamic acid at all but can utilize aspartic acid very slowly, it seems likely that an incompletely specific transaminase may be involved. Glutamic acid and aspartic acid transaminases have been found to function at lower levels on other amino acids. These mutants may completely lack glutamate transaminase activity while they retain aspartate transaminase activity, or they may have had the specificity and/or the configuration of the enzyme changed enough to allow it to function only partially and at a very low rate. However, further experimentation including specific assays for these enzymes will be necessary before definite conclusions can be drawn concerning the transaminases.

It has been shown often that deletions can arise which intrude simultaneously into bacterial and prophage chromosomes, or into genes controlling phage sensitivity and metabolic functions. In E. coli K-12, B, and C, mutations to resistance to phage T_1 are often associated with a requirement for tryptophan and are known to be due to deletions extending into the adjacent tryptophan region (80). More recently, the lambda prophage has been mapped by a series of deletions involving the galactose region (81). These studies were done using a method of mapping by deletion or multisite mutants. This method was first used by Benzer (82) for fine structure analysis of r_{II} mutants of the T_4 bacteriophage for E. coli. Mutants of this type behave as if they were completely lacking a part of the chromosome. The extent of the

deletion can be defined by crossing the deleted mutant with a series of single-site mutants whose mutational sites have already been mapped. Two multisite mutants whose deletions overlap will not be able to produce a wild type recombinant, but if there is no overlap between two deletion mutants then they may recombine in the region between the deletions and give rise to a wild type recombinant. By isolating a number of overlapping, multisite mutants and crossing them with each other, the presence or absence of wild type recombinants can be used to locate relative positions of the loci involved on the chromosome. This type of mapping experiment would be extremely useful with the mutants used in this study, but would require the isolation of additional mutants. A series of overlapping multisite mutants would give much valuable information concerning the relative positions of the apparently closely linked loci described earlier.

Data obtained in this study are in agreement with studies done by Green (65) in this laboratory. Both sets of data agree in the finding of two crossfeeding subgroups in the conversion of homoserine to cystathionine, suggesting the presence of at least two enzymatic steps. Green reported a similar subgroup found in the conversion of cystathionine into homocysteine. Only two mutants which possessed this defect were used in the present study, and both of these mutants belong to the same subgroup.

In both Sal. typhimurium (8) and E. coli (7,9) more than one enzyme has been implicated in the conversion of homoserine into cystathionine, as previously discussed. The intermediate formed in E. coli has been found to be O-succinylhomoserine (9,10,11), while O-acetylhomoserine is the intermediate in Neurospora (11,13).

Green (65) has obtained transduction data which indicate that a minimum of two enzymatic steps are involved in the conversion of cystathionine to homocysteine, and that these steps are coded for by closely linked loci or a single locus. However, only one enzyme was found for this step in E. coli (15).

The present study has indicated that there may be a close degree of linkage between the met 1 marker and that for phage sensitivity. This is in agreement with the findings of Green (65) using F116 phage.

Further work is needed before definite conclusions can be drawn from currently available genetic work done with P. aeruginosa. A larger number of mutants would be helpful in making comparisons between the work of different individuals. Enzyme assays of various groups of mutants would also be relevant to gaining insight into the linkage relationships and control mechanisms in P. aeruginosa, and would enable comparisons to be made with other organisms.

A comparison of data involving both crossfeeding and utilization of the methionine precursors would seem to indicate that the same pathway for the biosynthesis of methionine exists in P. aeruginosa as has been found in E. coli.

CHAPTER V

SUMMARY AND CONCLUSIONS

In this study a large group of mutants of Pseudomonas aeruginosa, originally selected for inability to utilize glycerol, was found to possess many other defects in addition to the one selected for. This group, PA-1-623 through PA-1-678, was found to consist of identical mutants. The cells had lost the ability to utilize glucose as a carbon source without supplementation of the medium with yeast extract. The required factor was found in the present study to be methionine.

The additional defects of this mutant group were found to be inability to utilize the following compounds as carbon sources: gluconate, mannitol, and glutamic acid. The organisms also were found to be resistant to phage specific for the wild type organism.

Evidence would appear to indicate that these mutants were produced by a single large deletion. Numerous point mutations seem unlikely to have produced so many identical mutants with the same requirements. Also, no spontaneous reversion rate has been observed at any time, and further treatment with mutagenic agents failed to produce any revertants.

A second series of mutants of the same organism, PA-1-92 through PA-1-97, was produced by treatment with a different mutagen and selected for methionine auxotrophy. These mutants have been shown to be identical in all respects to the first group, PA-1-623 through PA-1-678.

These cells also show a lack of reversion. The presence of two such groups adds evidence for the possibility that both groups are deletion mutants.

Growth studies with the available precursors of methionine (homoserine, homocysteine, and cystathionine) indicate that both the above mutant groups appear blocked in the conversion of homoserine to cystathionine. Two mutants in addition to these series also appear blocked in this step. Two additional mutants appear blocked in the conversion of cystathionine to homocysteine. No mutants were found which could not convert homocysteine to methionine.

Crossfeeding data indicate that there are two steps involved in the conversion of homoserine to cystathionine, and that the large group of organisms which lack this ability can be divided into two subgroups on the basis of these studies. Due to a lack of availability of O-succinylhomoserine for precursor studies, no growth data could be obtained indicating which mutants were blocked in each step on the basis of growth. However, crossfeeding data locates these steps. Only one member of this group, PA-1-27, appears to be blocked later in the sequence of the pathway than do all other members. PA-1-55 and PA-1-64 could not be utilized in such studies due to a high reversion rate.

Crossfeeding data produced further information regarding the two organisms unable to grow on cystathionine. These mutants would be expected to feed both groups blocked earlier in the sequence of synthesis, but this was not found. They were able to feed the groups blocked between homoserine and O-succinylhomoserine, but could not feed the organism blocked in the conversion of O-succinylhomoserine to cystathionine. For this reason these mutants may be lacking both

cystathionine-γ-synthase and β-cystathionase.

Only the mutant groups blocked in the conversion of homoserine to O-succinylhomoserine are resistant to phage, and only this group cannot utilize gluconate, glutamic acid, or mannitol. All other mutants used were not found to possess these defects. Therefore it is postulated that loci for utilization of glycerol, synthesis of homoserine succinylase, and phage resistance all are located adjacent to or near each other on the cell genome.

The PA-1-623 through PA-1-678 series has been shown to possess only a low, basal level of triose phosphate isomerase activity, and activity for L-α-glycerophosphate dehydrogenase is completely absent (1). Other glycerol mutants having normal characteristics in all other respects discussed retain dehydrogenase activity but have a low isomerase activity. Therefore, the dehydrogenase locus would seem to be located very near to the phage resistance locus, with the locus for the isomerase perhaps located on the opposite side of the dehydrogenase locus. Loci for utilization of gluconate, mannitol, and glutamate are thought to be located near this segment of the genome in an unknown order.

The resistance to phage exhibited by those mutants lacking the methionine enzyme 1 is thought to be due to some aberration in the cell wall structure, since the adsorption sites are located there in E. coli. Adsorption experiments have shown that the phage are not adsorbed to these mutants. Presumably an irreversible change has occurred in the structure of the cell wall or in the synthesis of some component required there, since none of these mutants have shown susceptibility to the wild type phage in any degree. None of the phage which were

isolated and found specific for these mutants have been able to cause lysis in any other type of mutant or in the wild type organism.

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APPENDIX A

ORIGIN OF MUTANTS USED IN THIS INVESTIGATION

Mutant strain	Origin
PA-1-623--678	Prolonged exposure to nitrosoguanidine on agar medium
PA-1-92--97	Treatment with ethyl methane sulfonate for 16 hours in liquid medium followed by a growth step in nutrient broth, then selection by carbenicillin and replica plating
PA-1-27, -55, -64	Three separate treatments with ethyl methane sulfonate in series, followed by penicillin selection and replica plating
PA-1-503--506	Short term exposure to nitrosoguanidine in liquid medium followed by a growth step, then penicillin selection and replica plating
Glycerol mutants A, C, D, E, F, 19, 20, 23	Short term exposure to nitrosoguanidine in liquid medium followed by a growth step, penicillin selection, and replica plating
PA-1-7	Treatment with acriflavin during growth followed by a growth step, and selection by exposure to Pϕ-6, then further screening with Group I phage

APPENDIX B

LIST OF BACTERIOPHAGES USED IN THIS INVESTIGATION AND THEIR ORIGINS

Phage strain	Origin
A. Mutant phage	
1) Group I Phage	
ϕ-631	Isolated in this investigation using PA-1-631 as the host
ϕ-632	Isolated in this investigation using PA-1-632 as the host
ϕ-634	Isolated in this investigation using PA-1-634 as the host
ϕ-639A	Isolated in this investigation using PA-1-639 as the host
ϕ-639B	Isolated in this investigation using PA-1-639 as the host
ϕ-640	Isolated in this investigation using PA-1-640 as the host
ϕ-641	Isolated in this investigation using PA-1-641 as the host
2) Group II phage	
ϕ-1	Host range mutant of ϕ-631
ϕ-2	Host range mutant of ϕ-639A
ϕ-3	Host range mutant of ϕ-639B
ϕ-4	Host range mutant of ϕ-634
ϕ-5	Host range mutant of ϕ-641
ϕ-6	Host range mutant of ϕ-632

Phage strain	Origin
2) Group II phage - continued	
ϕ-7	Host range mutant of ϕ-640
ϕ-8	Host range mutant of ϕ-631
ϕ-9	Host range mutant of ϕ-641
B. Stock wild type phage	
Pϕ-1	Donated by B. W. Holloway (originally F116 phage)
Pϕ-1.1	Derived from Pϕ-1
Pϕ-1.2	
Pϕ-2	Isolated from a wild type strain of *P. aeruginosa* isolated at Oklahoma State University
Pϕ-3	Isolated from a culture donated by the Oklahoma Medical Research Center
Pϕ-5	"
Pϕ-6	"
Pϕ-7	"
Pϕ-8	"
Pϕ-9	"
Pϕ-10	"
Pϕ-11	"
Pϕ-12	"
Pϕ-13	"
Pϕ-14	"
Pϕ-15	"
Pϕ-16	"
Pϕ-17	"

Phage strain	Origin
B. Stock wild type phage - continued	
Pϕ-18	Isolated from a culture donated by the Oklahoma Medical Research Center
Pϕ-19	"
Pϕ-20	"
Pϕ-21	"
Pϕ-22	"
Pϕ-23	"
Pϕ-24	"
Pϕ-25	"
Pϕ-26	"
Pϕ-27	"
Pϕ-28	"
Pϕ-29	"
Pϕ-30	"
Pϕ-31	"
F116-V-1-2	Clear plaque mutant of F116 isolated by B. Bruce in this laboratory
F116-V-2-2	"

VITA

2

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