GENETIC TRANSFER IN PSEUDOMONAS AERUGINOSA

By

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GENETIC TRANSFER IN PSEUDOMONAS AERUGINOSA

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CHAPTER I

INTRODUCTION

Three systems are known for genetic exchange in bacteria involving the transfer of deoxyribonucleic acid (DNA) from a donor to a recipient cell. They differ basically in the mode of DNA transfer. Transformation results from the incorporation of a free exogenous DNA fragment into a competent recipient cell. Conjugation involves the transfer of a large segment of DNA from donor to recipient through a connecting tube. Transduction is dependent upon enclosure of donor DNA by phage coat protein and subsequent injection of the DNA into a recipient cell. Direct cell-to-cell contact is therefore necessary only in conjugation.

Conjugation was first discovered by Lederberg and Tatum in 1946 in Escherichia coli K-12 (1). They found that the presence of a certain episome confers on a cell the ability to transfer genetic material to a cell lacking such an episome. The episome was referred to as the fertility or F factor; it may exist in two states: autonomous or incorporated. In the former, the cell is referred to as an F^+, and the episome may be transferred to recipient cells independently of the bacterial chromosome. In the latter, the cell is called an Hfr (for high frequency of recombination)

because during conjugation the transfer of bacterial chromosome is greatly promoted. Each of these male forms is capable of conversion to the other by mutation (one mutation in 10^6 cells), or conversion to the female form by loss of the F factor. However, the female (F^-) strains cannot mutate to the male state; they may become males only by acquiring the F factor from a male.

A schematic drawing of conjugation showing the transfer of the episome in either state is presented in Figure 1 (2).

In 1955, Holloway (3) reported a conjugating system for Pseudomonas aeruginosa which was later shown to be similar to the F^+ mating system of Escherichia coli (4). The infectious fertility factor associated with maleness was called pseudomonal fertility factor or FP.

Holloway and Fargie (5) found that the two fertility factors (F and FP) had similar characteristics. The transfer of FP is unidirectional; i.e., FP is transferred only from the FP^+ donor to the FP^- recipient. FP is transferred with varying degrees of efficiency depending on the particular FP^+ strain used. At the same time, F and FP were found to differ, in that FP is not destroyed by acridine, cobalt, or nickel [one exception has been noted with nitrogen half mustards (6)], nor has FP been shown to mutate to a stable Hfr form.

The conjugation system of P. aeruginosa has produced only low frequency mating (3, 4, 5, 7) which is similar to that in the $F^+ \times F^-$ mating system (8). However, Loutit and

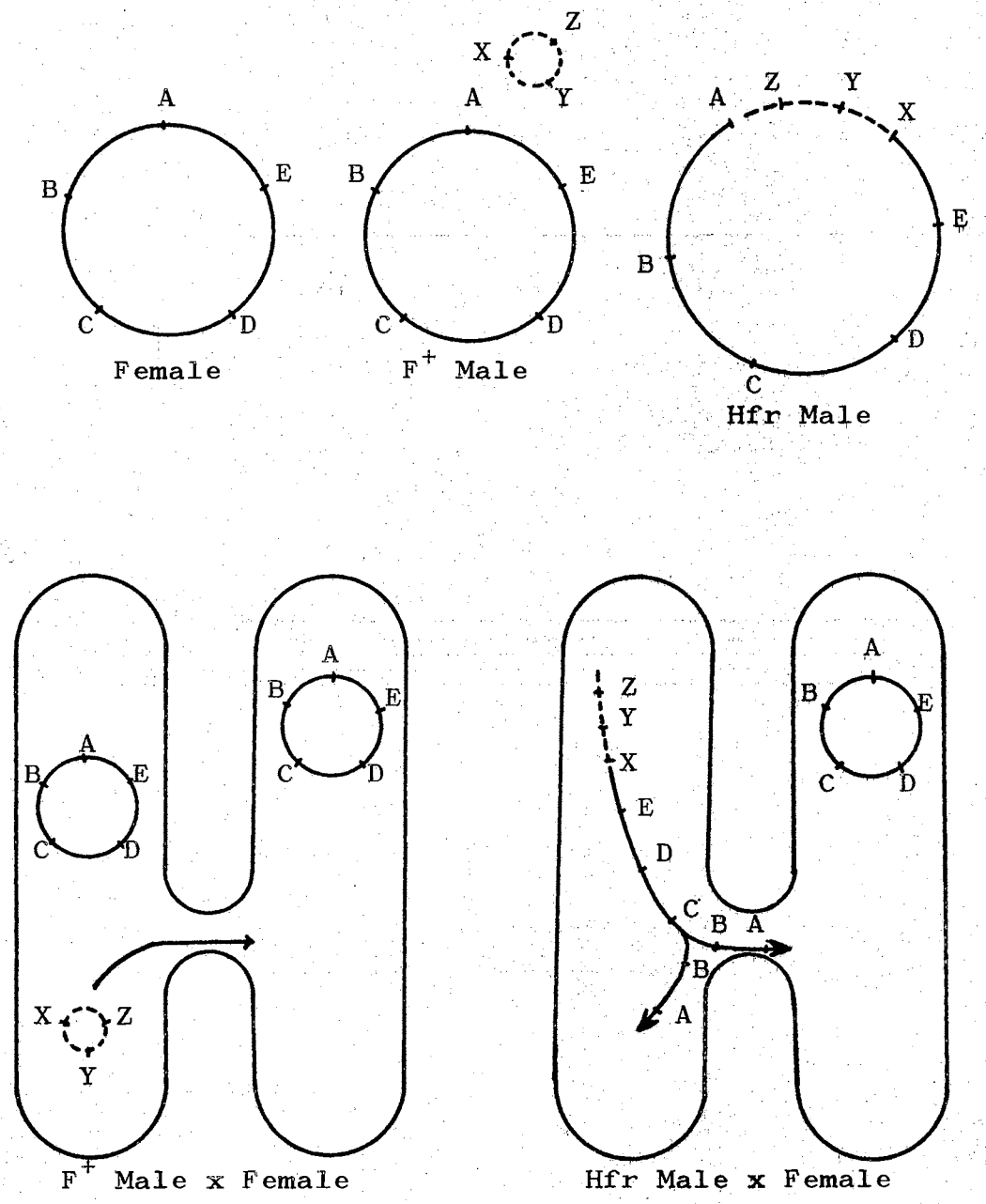


Figure 1. Schematic Drawing Showing Transfer of Episome in Two States by Conjugation

Pearce (9, 10) in 1965 reported that a greater efficiency of mating was obtained when parental cells were diluted to approximately 10^7 cells per ml. They found that mating was a variable event susceptible to changes in environmental conditions, which further complicated the already difficult analysis of the low-frequency system.

In part the following report describes modifications of methods previously used in hope of obtaining a low-frequency system that does not exhibit high variability but that is efficient and reliable. Since the best linkage data for E. coli have come from the use of high-frequency or Hfr donors, this author, despite negative results reported by Holloway and Fargie (5), also sought to isolate Hfr donor strains of P. aeruginosa.

An important feature of genetic analysis by conjugation is the selection for the inheritance of donor markers among recombinants; it is necessary to restrict the growth of both parental strains. A selective minimal medium may be used to prevent the growth of the auxotrophic recipient and antibiotics may be included to inhibit the growth of the sensitive donor. Only those cells which have received the necessary biosynthetic information from the donor (recombinants) will be able to grow.

Because analysis of a low-frequency conjugation system is difficult, transduction studies were also used to analyze linkage of methionine loci in P. aeruginosa. Zinder and Lederberg originally described transduction (11), a process

whereby bacterial genetic markers from the initial host cell are carried by phage particles to cells subsequently infected (12). For these markers to become permanently associated with the second cell line, genetic recombination must take place between the fragment of genetic material transferred and the host chromosome. Since this fragment represents only a small fraction of the entire chromosome, transduction is not useful for studying the over-all relationship of chromosomal markers. However, it is valuable for genetic fine structure analysis, such as intragenic mapping.

The technique of transduction has been widely exploited in the study of metabolic pathways. In Salmonella typhimurium, Demerec and Hartman (13), and Hartman (14) demonstrated that the groups of loci controlling tryptophan and histidine biosynthesis, respectively, were linked and ordered in a manner corresponding to the reaction sequences involved. Based on biochemical and genetic studies, transductional analysis was shown to be capable of distinguishing groups of mutants with blocks in different reaction steps in a pathway (15).

The value of a transducing system for mapping is determined by the length of chromosomal fragment transferred, which varies with the phage involved. Lennox (16), using phage P1 with E. coli, was able to demonstrate co-transduction of up to four markers. Yanofsky and Lennox (17) used the co-transducing properties of this phage in

their fine structure study of the loci concerned with tryptophan biosynthesis in E. coli.

In 1958 Loutit reported a generalized transducing system in P. aeruginosa (18). Culture filtrates of this organism were used to obtain genetic transfer within a single strain.

Transducing phages B3, P110, and F116 were isolated for P. aeruginosa by Holloway and Monk (19), and Holloway et al (20). Treatment of F116 with ultraviolet radiation led to the discovery that this phage could co-transduce linked bacterial markers at measurable frequencies (20, 21). These results indicated the suitability of this phage for a fine structure study of the P. aeruginosa chromosome.

The composite information from conjugation, transduction, and biochemical studies indicates that, in general, E. coli (17, 22, 23, 24), Salmonella typhimurium (25, 26, 27), Bacillus subtilis (28, 29), and Staphylococcus aureus (30) show clustering of loci affecting sequential steps in certain biosynthetic or degradative pathways. Such an arrangement does not seem to exist in Neurospora crassa (31, 32) and Saccharomyces cerevisiae (33). It has also been reported that many functionally related loci in P. aeruginosa are widely distributed throughout the genome. Linkage was not found within groups of mutants requiring histidine, leucine, arginine, tryptophan, methionine, or isoleucine-valine by Holloway, Hodgins, and Fargie (21). This report was confirmed in 1965 by Fargie and Holloway

(34) with cysteine, proline, adenine, and uracil auxotrophs tentatively added to the list. Mee and Lee (35), and Loutit and Marinus (36) reported non-linkage of histidine markers, while methionine markers were found by Calhoun and Feary (37) to be unlinked. There have been a few exceptions to this rule: two loci controlling the homoserine-threonine pathway and possibly two markers of the tryptophan pathway (34); four loci controlling the biosynthesis of isoleucine-valine (38); and nine loci involved in the biosynthesis of mandelate (39).

The difference in genetic organization between the two groups, those organisms with clustering of related genes and those without, is significant because of the type of genetic control involved in each. In the former, the unit of metabolic control is the operon (40), but in those with scattered loci, the means of control are not fully understood. It is not unlikely that the latter group are subject to another type of control mechanism or at least a modification of that found in the group with clustering. Demerec, in a 1964 review (26), pointed out that since P. aeruginosa and N. crassa show little clustering of genes and are considered to be lower and higher, respectively, than E. coli and S. typhimurium on the evolutionary scale, those organisms which developed the operon control system may have evolved as a side-branch in the evolutionary scheme.

The following work was undertaken to investigate the genetic systems in P. aeruginosa using both conjugation and

transduction methods in order to develop more efficient and reliable systems of genetic transfer. Linkage of related genetic markers was also investigated using primarily those from the methionine biosynthetic pathway. It was felt that by restricting the study to methionine-negative mutants of P. aeruginosa and by combining the results of biochemical tests with the transduction analysis, information might be obtained pertaining both to the steps involved in methionine biosynthesis in P. aeruginosa and the degree of linkage of methionine loci.

CHAPTER II

MATERIALS AND METHODS

A. Strains of Bacteria

The organism used throughout these studies was strain 1 of *P. aeruginosa* (designated PA-1), which was kindly supplied by Dr. B. W. Holloway, Monash University, Clayton, Australia. This is a female (FP^-) strain. Dr. Holloway also supplied the following cultures for use in conjugation studies: 1-36-8c, an adenine, leucine requiring (ade^- leu^-) male (FP^+) strain; 1-3 and 1-60, female (FP^-) strains requiring serine (ser^-) and isoleucine-valine (ilv^-), respectively. Other bacterial cultures which were tested but were not isolated by the author were obtained from Dr. Glen Bulmer, Medical Research Center, University of Oklahoma Medical School.

B. Strains of Phage

F116 was the bacteriophage used for transduction. This temperate phage was isolated and supplied to the author by B. W. Holloway.

C. Cultivation of Bacteria

The medium primarily used was a glucose minimal medium which was a modification of Robert's M-9 medium (41) and contained the following amounts per liter in distilled water: $Na_2HPO_4 \cdot H_2O$, 8.2 gm; KH_2PO_4, 2.7 gm; $MgSO_4 \cdot 7H_2O$, 0.4 gm; NH_4Cl, 1.0 gm; $FeSO_4$, 0.1% solution, 0.5 ml. Agar was added at a concentration of 20 gm/L when a solid medium was desired. The pH was adjusted to 7.0 before autoclaving. Glucose was used as the carbon and energy source at a final concentration of 5 gm/L; it was autoclaved separately as a 10 per cent (w/v) solution and added to the sterile salts solution. When the salts and agar were to be autoclaved together, the minimal time and temperature were utilized to avoid discoloration of the medium and precipitation of phosphates. Incubation of cultures was normally carried out at 37°C on a reciprocal shaker.

D. Treatment With Mutagens

1. N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG)

The mutagenicity of MNNG was first reported by Mandell and Greenberg (42). However, the actual procedure used was a modification of the method of Adelberg et al. (43) and is as follows: PA-1 cells were grown to 5-8 x 10^8 cells/ml in nutrient broth. A 10 ml sample was filtered through a 47 mm Millipore filter, pore size 0.45μ, and the cells were washed on the filter with 10 ml of

sodium citrate buffer, pH 6.0. Cells were suspended by placing the membrane in the original volume of citrate buffer containing 20µg MNNG/ml on a shaker for 2 hr at 37°C. The cell suspension was poured off, centrifuged, washed with physiological saline (0.85%), centrifuged and resuspended in 10 ml of nutrient broth. This cell suspension was placed on a shaker overnight at 37°C. Then the cells were centrifuged, resuspended in minimal salts containing no carbon or nitrogen source, and placed on a shaker at 37°C for 5 hr.

2. Ethylmethane Sulfonate (EMS)

A modification of the EMS procedure used by J. Nečásek et al. (44) to obtain auxotrophic mutants was used. The method is as follows: PA-1 cells were grown in nutrient broth on a shaker at 37°C. The cells were then diluted with nutrient broth to 3×10^9 cells/ml, and again diluted 1/3 with M-9 salts. Five ml of this cell suspension were centrifuged; the cells were washed twice and resuspended in 5 ml volumes of 0.067 M phosphate buffer, pH 7.2, and added to a flask containing 0.065 ml EMS. The flask was allowed to sit for 16 hr at room temperature before centrifuging and resuspending the cells in saline. To 6 ml of nutrient broth, a 0.2 ml inoculum of the treated cells was added, and these were allowed to grow to 10^9 cells/ml. Cells were then diluted with nutrient broth to 10^8 cells/ml. Six ml of diluted cell suspension were centrifuged and washed twice and resuspended in 6 ml volumes of M-9 salts (containing no

ammonia) plus 0.5% glucose, and placed on a shaker at 37°C for 3 hr to exhaust the amino acid pools.

E. Isolation of Mutants

1. MNNG

A 1/10 dilution was made of the 5-hr culture using glucose minimal medium containing 50,000 units of penicillin/ml. (The penicillin was obtained from Calbiochem of Los Angeles as Penicillin G, Potassium, U.S.P.) The penicillin was weighed and added directly to the medium. The culture was incubated overnight on the shaker at 37°C, then centrifuged, washed once and resuspended in an equal volume of 0.85% saline. Dilutions of 10^{-1}, 10^{-2}, and 10^{-3} were prepared in saline and 0.1 ml amounts of each dilution and of the undiluted suspension were spread with a glass rod on plates of glucose minimal agar plus 0.02% casamino acids (CAA). The plates were incubated at 37°C. The cell suspension was kept at 4°C until growth could be scored on the glucose plates. The dilution giving between 50 and 100 colonies per plate was noted and 20 plates of glucose minimal agar plus 0,02% CAA were inoculated by spreading 0.1 ml of the same dilution on the surface. Incubation was at 37°C until the colonies were 1-2 mm in diameter. The colonies were then replica plated by the velveteen pad technique of Lederberg and Lederberg (45) onto plates of glucose minimal agar and glucose minimal agar plus 0.02% CAA. Mutants were those which

formed colonies on glucose plus CAA but not on glucose alone. These were picked individually from plates containing CAA, and were checked again for growth on glucose and on glucose plus CAA.

2. EMS

Carbenicillin (α-carboxybenzylpenicillin) was dissolved in 0.05% glucose - M-9 medium and then added to the 6 ml of cell suspension to a final concentration of 50 μg/ml. (The carbenicillin was obtained from Beecham Research Laboratories, Piscataway, New Jersey). The culture was incubated for 7 hr. Dilutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} were prepared in sterile saline and 0.1 ml amounts of each dilution and of the undiluted suspension were spread on plates of glucose minimal agar plus 0.1% methionine. The cell suspension was kept at 4°C until growth could be scored. The dilution giving about 100 colonies per plate was noted and 0.1 ml of this same dilution was spread on each of 20 plates of glucose minimal agar plus 0.1% methionine. After incubation for 24 hr at 37°C, those plates were replica-plated onto glucose minimal medium and glucose minimal plus 0.1% methionine. Mutants, which grew only on the glucose minimal plus methionine plates, formed colonies only after 48 hr incubation. These colonies were picked individually and checked again on the two media.

F. Identification of Mutants

The glucose minimal medium described previously (section C) was modified to contain no nitrogen and only one-half the quantity of $Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4. This medium, containing 1.5% agar was poured in 10 ml portions into tubes and autoclaved. Cells were washed from a fresh (24 hr) nutrient agar slant with 2 ml of saline and 0.1 ml of this suspension was added to each tube of agar before pouring into petri dishes. Loopfuls (about 0.05 ml) of 2% amino acid solutions were placed at marked spots on the agar surface. Solutions containing groups of related amino acids were used first; then the members of the groups on which the mutants grew were checked individually in the same manner. Incubation at 37°C was for 72 hr. Identification was confirmed by growth in liquid medium (M-9 plus glucose) containing from 0.03% to 0.5% solution of the required amino acid.

G. Selection of Streptomycin-Sensitive Prototrophic Cultures

Cells were washed from a fresh nutrient agar slant with 2 ml of saline and 0.1 ml of this suspension was spread over the surface of glucose minimal agar and replicated onto glucose minimal agar and glucose minimal agar plus 100 μg streptomycin per ml. (The streptomycin was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio). Incubation was at 37°C for 72 hr. Cultures which formed

colonies on the glucose minimal but not on glucose minimal plus streptomycin were chosen. Colonies from glucose agar plates were picked individually and checked again on fresh media.

H. Selection of Streptomycin-Resistant Amino Acid Auxotrophs

The cultures used were amino acid-requiring mutant strains isolated previously. Cell suspensions were spread over glucose minimal agar plus 0.02% CAA and replicated onto glucose minimal agar and glucose minimal agar containing 100 μg streptomycin per ml and 0.02% CAA. Cells which formed colonies only on the streptomycin plates were picked and checked further.

I. Selection of Colymycin-Sensitive and Resistant Cultures

Cell suspensions were spread on nutrient agar and on nutrient agar plus 200 μg colymycin per ml. Sensitive cultures were picked from colonies on nutrient agar plates, and resistant cultures were picked from colymycin plates. All were checked further before making stock cultures. Colymycin (Colistin) was obtained from Warner-Chilcott, Laboratories Div., Morris Plains, N. J.

J. Selection of Double Resistance in Amino Acid Auxotrophs

Cells were washed from a fresh nutrient agar slant of a streptomycin-resistant culture with 2 ml of saline, and 0.1 ml of this cell suspension was spread on plates of nutrient agar plus 200 μg colymycin per ml. Likewise, 0.1 ml of a saline suspension of colymycin-resistant cells was spread on nutrient agar plus 100 μg streptomycin per ml. After incubation for 72 hr at 37°C, those colonies which appeared on either the streptomycin or colymycin plates were picked and checked further.

K. Grouping of Mutants According to Response on Methionine Precursors

Methionine-requiring (met^-) mutant cultures were grown on nutrient agar slants, and a washed cell suspension of each mutant was used to inoculate 15 ml of glucose minimal agar (1.5%). The tubes were mixed thoroughly, poured into sterile petri dishes and the agar allowed to solidify. Crystals of DL-homoserine, DL allo-cystathionine, DL-homocysteine thiolactone hydrochloride (Mann Research Laboratories, Division of Becton, Dickinson & Co., New York, N. Y.) and L-methionine (Nutritional Biochemicals Corporation, Cleveland, Ohio) were placed at marked points on the surface of the plates. After an incubation of 24 hr at 37°C, mutants were scored and grouped according to presence or absence of growth. These same mutants were tested in

liquid medium by adding 0.1 ml of the washed cell suspensions to 6 ml of glucose minimal medium, and glucose plus 33 mg/l of a specific biosynthetic intermediate or methionine. Optical density readings were taken at 12, 24, and 48 hr. Again mutants were grouped according to growth response on intermediates. The results of the two tests were compared.

L. Phage Titration

The Pseudomonas phage broth (20) used was composed of the following amounts per liter in distilled water: Difco nutrient broth, 8.0 gm; Difco yeast extract, 5.0 gm; NaCl, 5.0 gm. For platings, the bottom layer was made by adding 11 gm of Difco agar per liter of broth; plates contained 30 ml of agar. The top layer was semi-soft and contained only 6.5 gm agar per liter of broth.

The suspension to be titered was diluted serially in Pseudomonas phage broth. A 0.1 ml volume of each dilution (10^{-3}, 10^{-4}, 10^{-5}, 10^{-7}, 10^{-8}) was added to 2.5 ml of the top layer phage agar along with two drops of a heavy suspension of the sensitive bacteria. This was mixed and poured over the bottom layer. Incubation was at 37°C for 16-20 hr.

M. Phage Plate Stocks

Plate stocks of phage grown on both mutant and prototropic strains were prepared by a modification of the technique of Swanstrom and Adams (46) as follows: 0.1 ml of a

phage suspension containing approximately 1×10^6 plaque-forming units (PFU) per ml was plated with the desired cells as described above. After incubation for about 16 hr, 5 ml of phage broth were added to each plate and allowed to soak for 30 minutes. This was pipetted off and centrifuged to remove the cells. The supernatant was filtered through a Millipore filter (H.A., 0.45μ pore size), and titered on wild type cells. This method yields plate stocks of F116 with a titer of $10^{10} - 10^{11}$ PFU/ml.

N. Transduction

The transduction technique of Murphy and Rosenblum (47) for Staphylococcus aureus was used with slight modification. The plating medium was glucose minimal agar. Cells were washed from a fresh nutrient agar slant with 2 ml of saline and 0.1 ml of this suspension was spread on the agar. One drop of a phage plate stock was then placed on the surface at a marked point. At least six different plate stocks could be tested on each plate. Growth was scored after 72 hr at 37°C.

CHAPTER III

EXPERIMENTAL RESULTS

A. Conjugation Studies

1. Amino Acid Auxotrophs

Amino acid mutants of PA-1 were obtained through treatment with nitrosoguanidine and identified using the methods described in Chapter II. (See Appendix for a list of mutants.)

2. Conjugation With Donor Strain 1-36-8c

Using the above mutants and two FP^- amino acid mutants supplied by B. W. Holloway, conjugation studies were performed with an FP^+ strain, 1-36-8c, also donated by Holloway. Various modifications in Holloway's procedure were tried. Since 1-36-8c required both adenine and leucine, it was necessary to add adenine or leucine to the medium to prevent loss of recombinants due to the transfer of an adenine or leucine requirement. Optimal growth of 1-36-8c was achieved with 0.05% adenine and 0.2% leucine. Time of plating, temperature of incubation, and plating medium were varied, but numbers of recombinants were extremely low and variable. Such frequencies of

recombination were consistent with Holloway's work (3, 4, 5, 7).

3. Antibiotic Sensitivity of Strain 1-36-8c

Since antibiotic sensitivity is preferable for elimination of donor cells in a cross (as compared to nutritional requirements), an attempt was made to find an antibiotic which was effective against *P. aeruginosa* for use in subsequent studies. The FP^+ strain, 1-36-8c, was used to test various antibiotics. Cells were grown in nutrient broth for about 4 hr; 0.1 ml of the cell suspension was added to 2.5 ml of *Pseudomonas* phage soft agar. The cells and agar were thoroughly mixed and poured over 10 ml of solidified *Pseudomonas* phage bottom layer agar. The top layer was allowed to solidify, and commercially prepared antibiotic discs were placed on the surface (discs were obtained from the Stillwater Municipal Hospital). As can be seen from Table I, only colymycin definitely prevented growth of the FP^+ cells.

Colymycin was obtained, and the concentration necessary to inhibit male cells was determined first in liquid media and then in an agar medium. The FP^+ strain used, 1-36-8c, is ade^-, leu^-, and chloramphenicol-resistant (chl^R). When testing this organism in liquid defined medium, each tube contained M-9 salts plus 0.5% glucose, 0.05% adenine, and 0.2% leucine. Table II (a) indicates that 50 µg of colymycin per ml was sufficient to inhibit growth

TABLE I

THE EFFECTS OF VARIOUS ANTIBIOTICS ON PSEUDOMONAS AERUGINOSA 1-36-8c

No Clearing	Slight Clearing	Moderate Clearing	Complete Clearing
1. Altafur - 50 mcg	1. Demethylchortetracycline (declomycin) - 30 mcg	1. Sulfamethizole (thiosulfil) - 1 mg	1. Colistin (colymycin) - 10 mcg
2. Erythromycin estolate (ilosone) - 2 mcg	2. Oxytetracycline (terramycin) - 30 mcg		
3. Furazolidone (furoxone) - 100 mcg	3. Tetracycline (tetracyn) - 30 mcg		
4. Kanamycin (Kantrex) - 10 mcg			
5. Methicillin (staphcillin) - 5 mcg			
6. Nitrofurantoin (furadatin) - 100 mcg			
7. Nitrofurazone (furacin) - 100 mcg			
8. Novobiocin - 30 mcg			
9. Nystatin (myostatin) - 25 units			
10. Oleandomycin (matromycin) - 15 mcg			
11. Oxacillin (prostaphlin) - 1 mcg			
12. Penicillin G - 2 units			
13. Sulfamethoxy-pyridazine (madribon) - 50 mcg			
14. Triacetyloleandomycin (TAO) - 15 mcg			
15. Triple Sulfa (sulfadiazine, sulfamethazine sulfamerazine) - 50 mcg			
16. Vancomycin (vanococin) - 5 mcg			

mcg = micrograms
mg = milligrams
units = the measured amount of antibiotic necessary to cause destruction of the bacteria
1st name = common name
2nd name = trade name

TABLE II

DETERMINATION OF THE CONCENTRATION OF COLYMYCIN NECESSARY TO INHIBIT GROWTH OF SENSITIVE FP^+ CELLS

a) Colymycin, µg/ml in M-9, ade, leu	O.D. Reading at 24 hr
0	0.886
5	0.542
10	0.395
50	0.008
100	0.009

b) Colymycin, µg/ml in glucose minimal agar, ade, leu	Appearance of plate at 24 hr	Appearance of plate at 48 hr
0	Heavy growth over plate	Same
25	Heavy growth over plate	Same
50	Moderate growth over plate	Same
75	Isolated colonies over plate	Same
100	No growth	Isolated colonies
200	No growth	No growth

c) Colymycin, µg/ml in nutrient broth	O.D. Reading at 24 hr
0	0.653
50	0.011
100	0.009
200	0.011

O.D. (optical density) readings were taken at 540 nm.

completely. However, when tested in an agar medium a four-fold increase in the minimal inhibitory concentration was required. A washed cell suspension of 1-36-8c was spread on a plate of glucose M-9 agar containing adenine, leucine, 0.005% nutrient broth, and varying amounts of colymycin. Results are given in Table II (b).

Next, growth was checked in nutrient broth containing varying amounts of colymycin to determine whether the nutrients or the agar itself were responsible for the increase in inhibitory concentration. Table II (c) indicates that 50 μg of colymycin/ml was sufficient for inhibition of growth. Apparently, the agar is responsible for the lowered effectiveness of colymycin.

4. Selection of Prototrophic Streptomycin-Sensitive Cultures

Since 1-36-8c gave poor recombination frequencies, an attempt was made to find a new prototrophic FP^+ strain of P. aeruginosa.

Forty-nine strains of P. aeruginosa, previously obtained from Dr. Glen Bulmer, were tested for ability to grow on glucose minimal medium; 41 strains grew.

Before checking for the fertility factor, the strains found to be prototrophs were tested for sensitivity to streptomycin so that the males would have a contraselective marker for conjugation studies. The procedure used was

described in Chapter II. Six strains were streptomycin-sensitive, but four later became resistant.

5. Selection of Potential Donor Strains

Those strains which were streptomycin-sensitive (str^S) and prototrophic were tested for ability to transfer amino acid markers to the streptomycin-resistant (str^R) females. Transfer of the amino acid markers would indicate the presence of Hfrs in the str^S strains. The procedure used for conjugation studies was as follows: The two parental strains were grown separately overnight on a shaker in nutrient broth at 37°C. One ml of a str^S culture was added to a flask containing one ml of a str^R amino acid mutant plus 2 ml of nutrient broth. Control flasks contained one ml of a str^S or str^R culture plus three ml nutrient broth. The flasks were placed on a gently shaking water bath at 37°C for one hr. A 0.1 ml volume of each mixture was plated on glucose minimal agar containing 100 µg streptomycin/ml and 0.005% nutrient broth. Five days later the plates were scored. From the data shown in Table III, it appears that E-4 is an FP^+ strain. Conjugation with PA-1-54 (arg^-) and PA-1-76 (trp^-) gave relatively high numbers of recombinants. With PA-1-1, -27, -56, -73, and -75, there may have been a low frequency of recombination. Strain E-3 appeared to be inactive as a donor. The one amino acid mutant, PA-1-1, with which E-3 appeared to give recombinants had a relatively high rate of back mutation,

TABLE III

RECOMBINATION USING E-4 AND E-3 AS DONORS

FP⁻	No. of colonies on FP⁻ control	No. of colonies on E-4 x FP⁻	No. of recombinants	No. of colonies on FP⁻ control	No. of colonies on E-3 x FP⁻	No. of recombinants
PA-1-1	17	27	10	81	105	24
PA-1-17	2	0	0	2	2	0
PA-1-23	R	R	-	R	R	-
PA-1-27	11	17	6	18	19	1
PA-1-41	R	R	-	12	0	0
PA-1-54	13	40	27	25	25	0
PA-1-55	0	0	0	0	0	0
PA-1-56	2	11	9	16	9	0
PA-1-60	0	0	0	0	0	0
PA-1-63	62	25	0	-	-	-
PA-1-64	20	10	0	8	5	0
PA-1-72	1	1	0	1	3	2
PA-1-73	5	14	9	3	1	0
PA-1-75	1	8	7	11	7	0
PA-1-76	30	168+	138+	39	20	0
PA-1-83	R	R	-	R	R	-
PA-1-555	R	R	-	R	R	-
PA-1-586	R	R	-	R	R	-

E-4 and E-3 controls gave no growth. R indicates reversion of recipient.

therefore the results were questionable.

Strain E-4 was used for further conjugation studies, but to eliminate possible interference by mutation of E-4 to streptomycin resistance, another contraselective marker was necessary. The minimal inhibitory concentration of colymycin for this strain was determined on nutrient agar plates and found to be 200 μg/ml.

6. Isolation of FP^- Colymycin-Resistant Strains and Doubly Resistant Strains

At the same concentration of colymycin (200 μg/ml) that inhibited growth of E-4, $coly^R$ FP^- strains were isolated using the method described in Chapter II. These were then used to obtain double resistance (str^R $coly^R$) in the FP^- cells. The procedure used was given in Chapter II. It was necessary to modify this procedure to obtain the desired mutants of Holloway's strains 1-3 and 1-60, i.e., mutants resistant to 200 μg/ml colymycin. The cells were treated with UV radiation prior to plating since no spontaneous mutants resistant to this level of antibiotic were found. Doubly resistant mutants were obtained for the following amino acid auxotrophs:

1-3 (ser^-)	PA-1-27 (met^-)
1-60 (ilv^-)	PA-1-41 (pro^-)
PA-1-1 (?)	PA-1-54 (arg^-)
PA-1-17 (arg^-)	PA-1-55 (arg^-)
PA-1-23 (his^-)	PA-1-56 (his^-)

PA-1-60 (arg^-)	PA-1-75 (his^-)
PA-1-63 (arg^-)	PA-1-76 (trp^-)
PA-1-64 (met^-)	PA-1-83 (his^-)
PA-1-72 (arg^-)	PA-1-555 (leu^-, ilv^-)
PA-1-73 (arg^-)	PA-1-586 (leu^-, val^-)

7. Optimal Conditions for Conjugation

Conjugation studies were done with E-4 and str^R $coly^R$ amino acid auxotrophs using the procedure described previously, i.e., placing one ml of a freshly grown FP^+ culture with one ml of a freshly grown FP^- culture plus 2 ml of nutrient broth in a flask in a gently shaking water bath at 37°C for one hr. A 0.1 ml volume of each mixture was spread on a plate of glucose minimal agar containing 100 µg streptomycin/ml, 200 µg colymycin/ml, and 0.005% nutrient broth. (Hereafter, all media containing streptomycin and colymycin will be at these concentrations.) Initially, optimal cell concentrations were checked along with the effect that different ratios of FP^+ to FP^- cells might produce on recombination frequencies. Strains E-4 and PA-1-54, a combination known to produce recombinants (Table III) were grown in nutrient broth, and each was diluted to 1×10^{10}, 5×10^9, and 1×10^9 cells/ml. The latter two concentrations were tested at male to female ratios of 10/1, 5/1, 1/1, 1/5, and 1/10; however, the first concentration, 1×10^{10} cells/ml, was tested only at the ratios 10/1 and 1/10. After the proper amounts of cells were added to the

flasks to give the desired ratios, the remainder of the mating procedure was as described above. As can be seen from Table IV, it appeared that a cell concentration of 1×10^9 with 10 times more females than males gave the best recombination. This is in agreement with Loutit and Pearce (9); however, they obtained an even greater number of recombinants when the cultures were diluted to 1×10^7 cells/ml. Jacob and Wollman (48) reported an optimal female to male ratio of 20:1 for conjugation in E. coli. Holloway (5), for conjugation in P. aeruginosa, diluted both parents to 5×10^9 cells/ml, mixed equal volumes of the parental suspensions, and immediately plated 0.2 ml of this suspension. However, in later work (published after completion of this research), he used 10-20 males per female (6).

8. Attempted Isolation of Hfr Strains

Since the frequency of recombination obtained with E-4 was low, it seemed worthwhile to attempt to isolate an Hfr mutant of E-4. The procedure, that of Jacob and Wollman (49), was as follows: A 0.1 ml aliquot of a washed cell suspension of E-4 was spread on glucose minimal agar and allowed to grow overnight. Each plate of E-4 was replica plated onto an amino acid auxotroph freshly spread on glucose minimal agar plus streptomycin, colymycin, and 0.005% nutrient broth. Twenty different auxotrophic strains were used. The plates of E-4 used in replicating

TABLE IV

CONJUGATION WITH VARYING CELL CONCENTRATIONS AND DIFFERING RATIOS OF FP^+ TO FP^- CELLS

	No. of Colonies
E-4 control (0.1 ml of 1 X 10^{10} cell conc.)	0
PA-1-54 control (0.1 ml of 1 X 10^{10} cell conc.)	0
Both Cultures Diluted to 1 X 10^{10} cells/ml:	
FP^+/FP^- ratio	
10/1	0
1/10	0
Both Cultures Diluted to 5 X 10^9 cells/ml:	
FP^+/FP^- ratio	
10/1	85
5/1	117
1/1	0
1/5	21
1/10	7
Both Cultures Diluted to 1 X 10^9 cells/ml:	
FP^+/FP^- ratio	
10/1	94
5/1	58
1/1	47
1/5	78
1/10	TNTC

were stored at 4°C. From Table V, it can be seen that recombination took place between E-4 and the FP^- strains PA-1-41, -55, -60, and -72. When recombinant colonies were found on the amino acid mutant plates, the corresponding areas were located on the E-4 plates used for replicating, and a portion of the growth was removed to make fresh cultures on nutrient agar slants. These cultures should have higher concentrations of Hfrs since Hfrs are required for genetic transfer (49). To test for increased numbers of Hfrs, the mating procedure in nutrient broth was repeated. However, using the information gained earlier, the FP^+ and FP^- cultures were first diluted to 1×10^9 cells/ml and a 10/1 female to male ratio was used. Results are shown in Table VI. Recombination occurred when "Hfr" 1 was combined with Holloway's strain 1-3 or with PA-1-54; "Hfr" 5 with PA-1-54 and -72 (the latter was questionable due to a heavy background of very thin growth); "Hfr" 9 with PA-1-54; "Hfr" 13 with PA-1-54; "Hfr" 21 with PA-1-72 (again questionable); "Hfr" 33 with PA-1-23 and -54; and "Hfr" 45 with PA-1-23. (See Table VII for a summary.) It is interesting that the "Hfrs" were picked from areas that indicated a high frequency of recombination with mutants PA-1-41 (pro^-), -55 (arg^-), -60 (arg^-), and -72 (arg^-) in an earlier test; yet when these "Hfrs" were tested further, no recombination with these particular amino acid mutants was found. However, the marker transferred most frequently in all experiments was arginine.

TABLE V

CONJUGATION BETWEEN E-4 AND FP$^-$ AMINO ACID MUTANTS IN SEARCH FOR Hfr MUTANTS OF E-4

Mutant	No. of colonies on FP$^-$ control	No. of colonies E-4 X FP$^-$	No. of recombinants
PA-1-1 (?)	0	0	0
PA-1-17 (arg$^-$)	0	0	0
PA-1-23 (his$^-$)	0	0	0
PA-1-27 (met$^-$)	2	0	0
PA-1-41 (pro$^-$)	0	40	40
PA-1-54 (arg$^-$)	0	0	0
PA-1-55 (arg$^-$)	0	15	15
PA-1-56 (his$^-$)	0	0	0
PA-1-60 (arg$^-$)	0	18	18
PA-1-63 (arg$^-$)	0	0	0
PA-1-64 (met$^-$)	0	3	3
PA-1-72 (arg$^-$)	0	15	15
PA-1-73 (arg$^-$)	0	0	0
PA-1-75 (his$^-$)	0	0	0
PA-1-76 (trp$^-$)	0	0	0
PA-1-83 (his$^-$)	0	0	0
PA-1-555 (leu$^-$, ilv$^-$)	0	0	0
PA-1-586 (leu$^-$, val$^-$)	1	R	-
1-3 (ser$^-$)	0	0	0
1-60 (ilv$^-$)	0	0	0

E-4 control had no growth. R indicates reversion of the recipient.

TABLE VI

REPEATED CONJUGATION IN SEARCH OF E-4 Hfr MUTANTS

"Hfr"

	1	2	5	9	13	17	21	25	29	33	37	41	45	47
PA - 1 -														
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	2	0	0	0	0	0
23	0	0	0	1	0	0	0	0	0	114	6	0	30	0
27	0	0	0	9	7	0	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	5	0	0	0	0	0	0	0
54	20	0	102	60	16	6	0	0	9	22	3	0	0	2
55	7	6	0	0	0	0	0	0	0	0	0	0	0	0
56	0	0	0	0	0	0	0	0	0	0	0	0	0	0
60	1	0	0	0	0	0	0	0	0	0	0	0	0	0
63	0	0	0	0	0	0	0	0	0	0	0	0	0	0
64	0	0	0	0	0	0	0	0	0	0	0	0	0	0
72	0	0	15	0	0	0	15	0	R	R	R	0	0	0
73	0	0	0	11	0	0	0	0	0	0	0	0	0	0
75	0	0	0	0	0	0	0	0	0	0	0	0	0	0
76	0	0	0	0	0	0	0	0	0	0	0	0	0	0
83	0	0	0	0	0	0	0	0	0	0	0	0	0	0
555	0	0	0	0	0	0	0	0	0	0	0	0	0	0
586	2	R	R	R	R	R	R	R	R	R	R	R	R	R
1-3	45	0	0	0	0	0	0	0	4	0	0	0	0	4
1-60	0	0	0	0	0	R	R	R	R	R	R	0	0	0

The numbers above are corrected for controls. R indicates reversion of the recipient.

TABLE VII

ATTEMPTED ISOLATION OF Hfr STRAINS

"Hfr"	Marker Transferred	No. of Recombinants
1	ser (1-3)	45
1	arg (PA-1-54)	20
5	arg (PA-1-54)	102
5	arg (PA-1-72)	15
9	arg (PA-1-54)	60
13	arg (PA-1-54)	16
21	arg (PA-1-72)	15
33	his (PA-1-23)	114
33	arg (PA-1-54)	22
45	his (PA-1-23)	30

Since "Hfrs" 5, 9, and 13 gave relatively high frequencies of recombination, an attempt was made to purify the "Hfrs" further, again using indirect selection. The procedure was repeated, replicating the "Hfrs" 5, 9, and 13 onto freshly spread plates of PA-1-54. With each isolation from a plate and replication onto $F^{-}P^{-}$ cells, progressively higher numbers of recombinants should be obtained. Results after incubation for one week are indicated in Table VIII (a). "Hfrs" 5 and 9 gave greatly decreased numbers of recombinants in comparison with the previous test, while "Hfr" 13 produced about the same number. Nevertheless, corresponding recombinant areas on the E-4 plates were again picked, the cells grown, and the procedure repeated. It can be seen from Table VIII (b) that no actual Hfr mutants were isolated.

A last attempt at isolating Hfrs was made. E-4 "Hfrs" 1, 5, 9, 13, 21, 33, and 45 were grown on nutrient agar slants and 0.1 ml of a washed cell suspension of each was spread over glucose minimal agar, exposed to ultra-violet light (15 watt Sylvania germicidal lamp) for 20 sec at 40 cm, and then incubated in the dark. Later, these cultures were replica plated onto those amino acid mutants which had given relatively high numbers of recombinants in a previous experiment. (See Table VII.) No colonies were found on the plates with the exception of one on the "Hfr" 33 X PA-1-54 cross. This inability to obtain Hfrs was shared by Holloway and Fargie (5) as well as Loutit (36). It

TABLE VIII

FURTHER ATTEMPTS AT ISOLATION OF Hfr STRAINS

a) Recombination After First Replica Plating

"Hfr"	No. of colonies on "Hfr" X PA-1-54	No. of Recombinants
5	0	0
5	1	1
9	1	1
9	2	2
13	9	9
13	12	12

Control plates for Hfr strains and PA-1-54 had no colonies.

b) Recombination After Second Replica Plating

"Hfr"	No. of colonies on "Hfr" X PA-1-54	No. of Recombinants
5_1	1	1
9_1	4	4
9_2	1	1
9_3	1	1
13_1	2	2
13_2	0	0
13_3	0	0
13_4	1	1

Control plates for Hfr strains and PA-1-54 had no colonies.

was concluded that Hfrs, if present, may be highly unstable relative to those in the E. coli system.

B. Transduction Studies of Methionine Biosynthesis

Since Hfr strains had not been isolated, detailed mapping studies could not be performed using conjugation. Therefore, it was decided to employ a different system of genetic transfer, transduction, and the methionine biosynthetic pathway was chosen for further study.

1. Isolation and Classification of Mutants

Additional methionine auxotrophs were isolated, using a different mutagen, ethylmethane sulfonate, for which the procedure was given in Chapter II. These mutants and the previously isolated methionine auxotrophs (a total of 27) were then grouped on the basis of growth on methionine and intermediates in the synthesis of methionine. Mutants were grown on nutrient agar slants, and 0.1 ml of a washed cell suspension of each mutant was used to inoculate 15 ml of melted glucose minimal agar. These were mixed well, poured, and allowed to solidify. Crystals of homoserine, cystathionine, homocysteine, and methionine were placed in marked areas on the surface of the plate. From the results shown in Table IX, there appeared to be two groups of mutants: PA-1-27, PA-1-64, PA-1-91, PA-1-93, and PA-1-95 with blocks before cystathionine, and PA-1-503, PA-1-506, PA-1-521, and PA-1-553 with blocks before homocysteine.

TABLE IX

GROWTH OF MUTANTS ON METHIONINE AND INTERMEDIATES
(SOLID MEDIUM)

Mutant	Homoserine	Cystathionine	Homocysteine	Methionine
PA-1-27	---	+	++	+++
PA-1-64	---	++	++	+++
PA-1-91	---	++	---	+++
PA-1-93	---	++	---	+++
PA-1-95	---	++	---	+++
PA-1-503	---	---	++	+++
PA-1-506	---	---	++	+++
PA-1-521	---	---	++	+++
PA-1-553	---	---	++	+++

--- no growth
+ slight growth
++ moderate growth
+++ heavy growth

Plates contained no back mutation

Growth of mutants on methionine and intermediates was checked further in liquid medium. A 0.1 ml volume of washed cell suspension was added to glucose (0.5%) minimal medium containing 0.003% of an intermediate or methionine. Optical density readings were made at 12, 24, and 48 hr. Results, shown in Table X, agree with those on solid medium except that PA-1-91, PA-1-93, and PA-1-95 apparently grew slightly on homocysteine. The slight growth was not detectable by the auxanographic method.

The methionine pathway in E. coli is given in Figure 2. It is not known whether this pathway is the same in P. aeruginosa, but from the information above, the intermediates appear to occur in the same order.

Met^- mutants were then tested for cross-feeding. Several methods were tried.

Cells were grown on nutrient agar slants, and a small inoculum was removed and streaked across plates of glucose (0.5%) minimal agar containing 0.0025% CAA. One mutant was streaked vertically down the plate, while the other mutants were streaked horizontally with care taken not to touch the inoculum of the other mutants. This method was described by Hayes (50). All mutants were tested in all possible combinations using the following pattern.

TABLE X

GROWTH* OF MUTANTS ON METHIONINE AND INTERMEDIATES
(LIQUID MEDIUM)

Mutant	Homo-serine	Cysta-thionine	Homo-cysteine	Meth-ionine	Control
PA-1-27	0.046	0.233	0.678	0.846	0.074
PA-1-64	0.039	1.059	0.770	0.912	0.028
PA-1-91	0.030	0.292	0.192	0.699	0.036
PA-1-93	0.026	0.240	0.097	0.663	0.032
PA-1-95	0.026	0.308	0.149	0.653	0.029
PA-1-503	0.016	0.032	0.710	1.000	0.016
PA-1-506	0.030	0.036	0.688	0.969	0.027
PA-1-521	0.016	0.022	0.620	0.878	0.027
PA-1-553	0.018	0.053	0.678	0.912	0.040
PA-1 wild type	0.770	0.870	0.886	0.903	0.770

Incubation was at 37° C.

*O.D.(readings taken at 48 hr at 540 nm.)

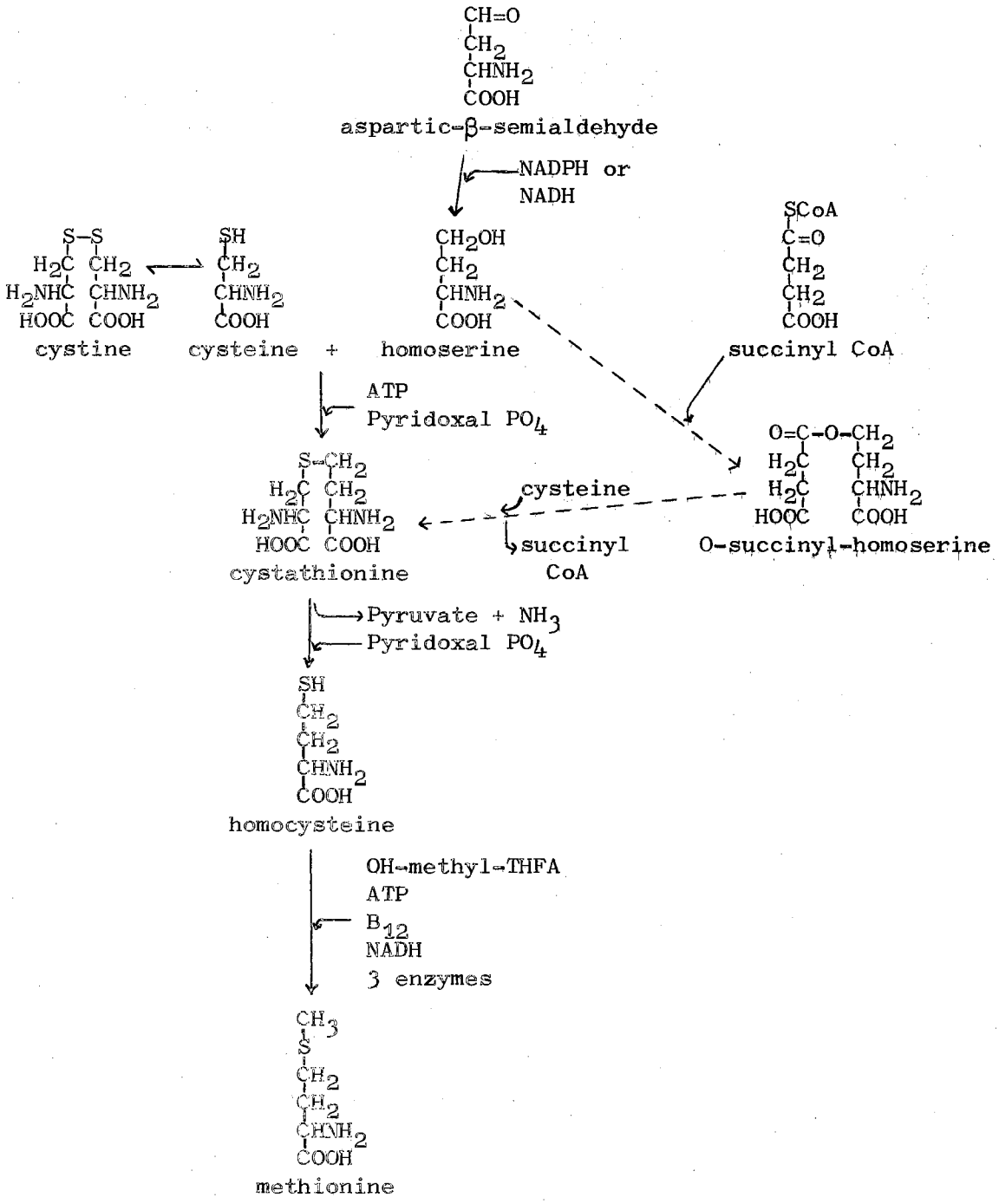
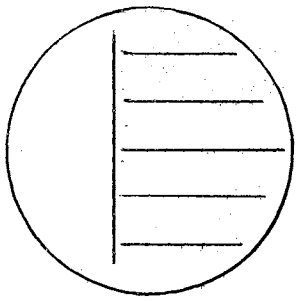


Figure 2. Methionine Pathway in Escherichia Coli

Diagram of Cross-feeding



The theory involved is as follows: each mutant may excrete into the medium the intermediate before its own block. The intermediate is then available to the other mutants, but only those mutants which are blocked before the intermediate are able to use it, and thus grow. Therefore, any mutant which can feed another will have a block in its pathway after that in the mutant which can be fed. From these data, an idea of the order of steps in a pathway can be obtained. However, poor results were obtained with this technique; mutants that did not show back mutation showed no cross-feeding.

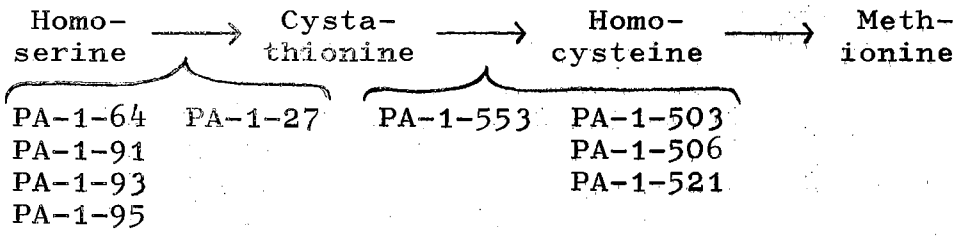
Met^- mutants were again checked for cross-feeding using the procedure of Pearce and Loutit (38) by first streaking for isolated colonies and then emulsifying a single colony in 1 ml of saline. Mutants were streaked as before on the same medium. Again no cross-feeding was obtained.

Fifteen ml of melted glucose minimal agar (1.5% agar) containing 0.0025% nutrient broth were seeded with 0.1 ml of a washed cell suspension, according to the procedure of Fargie and Holloway (34). This was mixed thoroughly and

poured into a sterile petri dish. Once it had solidified, drops of washed cell suspensions of the other mutants were spotted on the surface. Thus, feeding of one mutant by another could be detected as growth of either the surface cultures or the embedded cultures. Again, results were poor.

D. H. Calhoun (personal communication) had been successful with cross-feeding in Pseudomonas by inoculating 20 ml of melted glucose minimal agar (1.5%) containing 0.1 µg methionine/ml, or 0.025% nutrient broth and 0.005% yeast extract, with 0.5 ml of washed overnight culture, allowing to solidify, and spotting, as above, with other mutants. This differs from Fargie and Holloway's procedure in the amount of nutrient broth and cell concentration added (both greatly increased). This procedure was followed using glucose minimal agar plus 0.1 µg methionine per ml to test met$^-$ mutants. The results obtained are shown in Table XI.

From cross-feeding data as well as growth on intermediates, it is believed that the most probable arrangement of the mutants according to their metabolic blocks is that shown below.



Growth response to the available intermediates in the pathway is the most unequivocal basis for classification of

TABLE XI

CROSS-FEEDING OF METHIONINE AUXOTROPHS

	Fed By PA-1-								
	503	506	521	553	27	64	91	93	95
503	-	-	-	-	-	-	-	-	-
506	-	-	-	-	-	-	-	-	-
521	-	+	-	-	-	-	-	-	-
553	+	+	+	-	-	-	-	-	-
27	+	+	+	+	-	-	-	-	-
64	±	±	+	+	+	-	-	-	-
91	+	+	+	±	±	-	-	-	-
93	+	+	+	±	±	-	-	-	-
95	+	+	+	±	±	-	-	-	-

\- no growth

± slight growth

\+ moderate growth

No back mutation occurred on plates

mutants requiring methionine for growth. These data (given in Table X) allowed separation of the mutants isolated and used in the present study into two groups, one capable of growth on cystathionine, homocysteine, and methionine and a second which grew only on cystathionine and methionine. The first group is, therefore, blocked in the conversion of homoserine to cystathionine and the second group in the succeeding step, the conversion of cystathionine to homocysteine.

The cross-feeding data are in complete agreement with the division of the mutants into the two major groups. However, cross-feeding data indicate further division of these groups into sub-groups. Within the group blocked after homoserine, PA-1-27 feeds the other four mutants, PA-1-64, -91, -93, and -95. Within the second group, only the position of PA-1-521 is questionable, due to its apparent ability to be fed by PA-1-506. However, since PA-1-503, -506, and -521 all feed PA-1-553, it seems most reasonable to place PA-1-521 with the other two (i.e., with PA-1-503 and -506).

2. Grouping of Mutants by Transduction

As has been stated earlier, transduction is one method of genetic exchange in bacteria. Since it transfers only small segments of bacterial DNA, it can be used in fine structure mapping and elucidating metabolic pathways.

Plate stocks of F116 phage were prepared on the various

mutants. However, several mutants (PA-1-64, -91, -93, -95) did not produce plaques. The experiment was run repeatedly with the same results. Another phage known to produce plaques on P. aeruginosa (PΦ-25) was tested on these mutants with no plaques resulting. Transduction was carried out with all the possible combinations of the phage-sensitive mutants, using each as donor and as recipient. Each mutant was also treated with phage from the wild type strain.

The transduction technique, described in Chapter II, gave the results shown in Table XII. Conclusions regarding the linkage of methionine loci are based on comparisons of numbers of transductants obtained in wild type x mutant and mutant x mutant crosses (21). Data indicate that all the methionine mutants are linked. Phage propagated on PA-1-503 and -506 and used to transduce PA-1-27 give a few transductants indicating that perhaps PA-1-503 and -506 are not as closely linked to PA-1-27 as are the other mutants, PA-1-521 and -553. This corresponds to cross-feeding data. It is unfortunate that transduction studies could not be done with the other met^- mutants (PA-1-64, -91, -93, and -95) for which data on cross-feeding and growth requirements were obtained.

TABLE XII

TRANSDUCTION IN METHIONINE MUTANTS

Donor \ Recipient	PA-1-27	PA-1-503	PA-1-506	PA-1-521	PA-1-553
PA-1-27	0	0	0	0	0
PA-1-503	6	0	0	0	0
PA-1-506	5	0	0	0	0
PA-1-521	0	1	0	0	0
PA-1-553	0	0	0	0	0
PA-1 wild type	50	57	52	75	44

No back mutation occurred on plates.

Numbers given are averages of several tests.

CHAPTER IV

DISCUSSION

Present concepts of genetic exchange systems and genetic control mechanisms are based primarily on the work done in *Escherichia coli*. It is of importance to investigate and develop efficient genetic transfer systems for *P. aeruginosa*. With these, the linkage relationships of genetic markers and the corresponding control mechanisms can be investigated, thus allowing a comparison between members of two unrelated bacterial families.

Conjugation studies were first undertaken using Holloway's mating strains and several amino acid auxotrophic FP^- strains in hopes of improving techniques. Temperature and time of incubation as well as plating media were varied with extremely inconsistent results. When colonies did appear, they frequently were revertants. When using the same procedures and strains as Holloway, results similar to his were never obtained. For this reason, it is felt that either the strain was incorrectly identified as FP^+, or had lost the FP factor. Several strains were then tested with known recipient strains for ability to transfer prototrophic markers.

Modifications of methods of conjugation were undertaken

and it was found that diluting the parental cells to 10^9/ml in a ratio of 10 females per male gave the greatest number of recombinants. A comparison of the frequencies of recombination during conjugation in P. aeruginosa obtained in the present study with those reported by Holloway and Loutit is as follows: Holloway (3) reported 125 recombinants/10^9 parental cells in a strain 1 x strain 2 cross which is equivalent to one recombinant/4×10^6 FP^+ or FP^- cells. With strain E-4, in the present study, one recombinant was formed per 8×10^4 FP^+ cells (using figures taken from Table VII). This is, at best, 50 times greater than the frequency reported by Holloway. When crossing strain 1 with strain 1, Holloway and Jennings (4) obtained one recombinant per 10^7 FP^+ or FP^- cells. However, Loutit and Pearce (9) reported the highest frequency of recombination (using a strain 1 x strain 1 cross) with one recombinant/500 FP^+ or FP^- cells. This is much greater than the frequency for the $F^+ \times F^-$ crosses in E. coli which has been reported as one recombinant per 10^4 or 10^5 parental cells (8). Loutit et al. (51) further increased the recombination frequency to a maximum of 1% through the use of nitrate in nutrient broth, and by using a second subculture of FP^+ cells in a nitrate medium. The frequency at which recombinants are formed in the P. aeruginosa mating system is very low relative to the maximum frequency for crosses employing an Hfr in E. coli (one recombinant/10 Hfr). Until strains comparable to the Hfr strains are isolated for

P. aeruginosa, efficient mapping of large segments of the genome will not be possible.

In the course of this investigation, several observations were made which are of particular interest because most strains used in screening for donors were clinical isolates. When searching for an antibiotic contraselective marker, it was observed that a fourfold increase in colymycin concentration was necessary in nutrient agar to obtain absolute killing of the donor cells. A possible explanation for this phenomenon came through the work of Hanus, Sands, and Bennett (52) who tested antibiotic activity in the presence of washed and unwashed agar as compared to activity in nutrient broth. The minimal inhibitory concentrations of certain antibiotics, such as streptomycin, were found to be adversely affected by agar. This inhibition of antibiotic activity was believed to be caused by negatively charged acid and sulfate groups in agar which reacted with the basic groups of the antibiotics. Other factors which were shown to reduce antibiotic effects were: divalent metalic cations such as calcium and magnesium bound to agar, and traces of growth and stimulating factors in agar which alter the sensitivity of an organism to an antibiotic. The use of washed agar often produced minimal inhibitory concentrations of antibiotics approaching those in nutrient broth. In a recent paper by Kunin and Edmondson (53), zones of inhibition by colymycin and other antibiotics in various media were increased by replacing

agar with agarose, a neutral polysaccharide extract of agar which contains less than 0.5% sulfate. Protamine and toluidine blue were also found to reverse the binding of constituents in agar to antibiotics.

When checking for prototrophic cultures in P. aeruginosa, it was found that of 49 strains tested, 41 were prototrophic. Only 6 of these were str^S while the other 35 were str^R. The high number of str^R cultures was not surprising since all were isolated in hospitals or clinics.

Evaluation and interpretation of data relevant to linkage of methionine loci obtained in this study is difficult because the methods employed for obtaining data are variable in their reliability. Cross-feeding in P. aeruginosa was difficult to obtain (as shown by the many techniques studied), and, once obtained, was difficult to score reliably. For this reason, PA-1-521 could be grouped either with PA-1-553 or PA-1-503 and -506. It was felt that PA-1-521 appeared to constitute a third sub-group in the cystathionine-homocysteine block only because of this scoring difficulty. The inability of the auxotrophs to cross-feed may be due to slow excretion coupled with a sensitive feedback inhibition control mechanism such that a low internal level of the intermediate would be reached rapidly and would immediately inhibit further production. Autolysis of the cell may be essential for release of intermediates.

No difficulties were encountered with growth studies on

intermediates or with transduction and the resulting data are felt to be more reliable than those obtained with cross-feeding.

In these studies, close linkage was found between all the mutants which could be used in transduction studies. These include at least two loci in the methionine biosynthetic pathway. A more detailed look at all the information gathered on this pathway in P. aeruginosa would be helpful; however, due to the difficulties in methods and mutants studied, only limited comparisons can be made among the results reported here, the work of Holloway et al. and that of Calhoun and Feary. The following diagram may be helpful in interpreting the data reported for all the studies. The numbers refer to the position of the metabolic block for groups of mutants identified by growth response; i.e., mutants able to grow on any intermediate following the block.

$$\xrightarrow{1}\ \text{homo-serine}\ \xrightarrow{2}\ \text{cysta-thionine}\ \xrightarrow{3}\ \text{homo-cysteine}\ \xrightarrow{4}\ \text{meth-ionine}$$

In the present study, two cross-feeding sub-groups were found for both group 2 and group 3. Calhoun and Feary (37) identified mutants belonging to groups 2 and 3, but reported no cross-feeding data for them. Fargie and Holloway (34) reported cross-feeding of mutants within a single group which included both group 1 and 2 above. Therefore, no other cross-feeding data are available for comparison with those of the present study.

Calhoun and Feary (37) reported a single linkage group for mutants of group 3. This agrees with the finding of linkage between the two sub-groups with this group. Holloway found no linkage between mutants of his cross-feeding sub-groups. However, these were not sufficiently identified to determine whether they correspond to other reported groups. Calhoun and Feary (37) reported that groups 1 and 2 are not linked. This author's data show linkage between one sub-group of group 2 and both sub-groups of group 3. Calhoun and Feary (37) found no linkage between group 2 and 3. All these data may be consistent if Holloway's sub-groups correspond to Calhoun and Feary's sub-groups 1 and 2 and if all the mutants of Calhoun and Feary's group 2 correspond to one of the sub-groups found in the present study. In the absence of more complete data on the location of mutants studied by others, it is impossible to compare results more closely.

The occurrence of two sub-groups with a block between homoserine and cystathionine, with one cross-feeding the other, would seem to suggest the presence of at least two enzymatic steps. More than one enzyme concerned with the formation of cystathionine from homoserine and cysteine have been indicated in S. typhimurium (54) and E. coli (55, 56). Strong evidence has been produced that O-succinylhomoserine is the intermediate formed in E. coli (56, 57). (See Figure 2.) The reaction involving two steps is as follows:

$$\text{homoserine} + \overset{\text{Succinyl CoA}}{\longrightarrow} \text{O-succinylhomoserine} \xrightarrow[\text{glucose, ATP}]{\text{cysteine}}$$

cystathionine + succinate. O-acetylhomoserine is the intermediate formed in Neurospora at this step (57, 58). A similar reaction could be involved in P. aeruginosa with either of these or a related compound as an intermediate.

Transduction data indicated that a minimum of two enzymatic steps involving the conversion of cystathionine to homocysteine are coded for by closely linked loci or a single locus. In E. coli, one enzyme, cystathionine synthetase, simultaneously cleaves the link between the sulfur atom and the carbon atom of the three carbon chain of cystathionine and deaminates the three carbon fragment to form homocysteine, pyruvate, and ammonia (59). However, before the presence of one enzyme was confirmed, the possibility of two other pathways involving two enzymes each was studied. These possible pathways of cystathionine degradation were:

$$\text{cystathionine} \xrightarrow[\text{synthetase}]{\text{cystathionine}} \text{homocysteine} + \text{serine}$$

$$\text{serine} \xrightarrow[\text{deaminase}]{\text{serine}} \text{pyruvate} + NH_3$$

or

$$\text{cystathionine} \xrightarrow[\text{synthetase}]{\text{cystathionine}} \text{homocysteine} + \text{alanine}$$

$$\text{alanine} \xrightarrow[\text{deaminase}]{\text{oxidative}} \text{pyruvate} + NH_3$$

The cross-feeding data obtained in the present study indicate that in P. aeruginosa this rather complex reaction may involve more than one enzyme.

A considerable amount of additional work is needed before the methionine pathway in P. aeruginosa can be defined and the loci mapped. Valid comparisons cannot be made on the independent studies reported to date due to limited numbers of mutants used in each case. It would be desirable to attempt isolation of additional mutants using various mutagenic treatments to increase the probability of finding mutants from other growth response groups. Mapping might be possible using recent advances in conjugation techniques (36, 51, 60).

The marker for F116 sensitivity may be closely linked to the methionine marker, or the proposed intermediate may be essential in the synthesis of the F116 attachment site. If F116 sensitive mutants analogous to the F116 resistant mutants cannot be found, another phage might be used for transduction studies. The potential for transducing these mutants would allow one to look for abortive transductants as evidence against a one-enzyme step.

Enzyme assays of the various mutant groups would aid in classifying the mutants as defective in structural genes or in control genes since these may or may not map in the same region. All of this would be relevant to gaining an understanding of the linkage relationships and control mechanisms in P. aeruginosa.

CHAPTER V

SUMMARY AND CONCLUSIONS

This investigation included attempts to develop efficient genetic transfer systems in P. aeruginosa, and a study of the linkage relationships of methionine genetic markers. Conjugation studies with known FP^+ and FP^- strains gave negative results; therefore, a new FP^+ donor strain was sought and found, E-4. Attempted isolations of an Hfr strain of this donor using the procedure of Jacob and Wollman (49), however, were fruitless. Optimal conditions for conjugation using the double antibiotic-sensitive E-4 donor and double antibiotic-resistant amino acid auxotrophs were found to be: overnight growth of the two parental strains in nutrient broth at 37°C on a shaker; dilution of each parent with nutrient broth to 1×10^9 cells per ml; use of a 1 to 10 ratio of males to females; very gentle shaking in a water bath at 37°C for 1 hr; plating of 0.1 ml of the mixture on glucose minimal agar containing 100 µg streptomycin/ml, 200 µg colymycin/ml, and 0.005% nutrient broth.

The frequency of recombination without an Hfr strain was felt to be too low for detailed mapping studies; therefore, the transfer of genetic material by transduction was

investigated with emphasis on the methionine biosynthetic pathway. Methionine mutants were obtained by treating the wild type parental organism, P. aeruginosa strain 1, with nitrosoguanidine or ethylmethane sulfonate, and subsequent screening with penicillin or carbenicillin and the Lederberg replica plating technique (45).

The methionine-negative mutants were first characterized by growth response on the available intermediates and methionine both in liquid and on agar. Intermediates tested were: homoserine, cystathionine, and homocysteine thiolactone hydrochloride. The grouping obtained by growth response was as follows:

1. PA-1-27, -64, -91, -93, and -95. These mutants are able to utilize cystathionine, homocysteine, and methionine, and thus contain blocks which prevent the conversion of homoserine to cystathionine. These were further subdivided on the basis of cross-feeding, with PA-1-64, -91, -93, and -95 all being cross-fed by PA-1-27.
2. PA-1-503, -506, -521, and -553. These mutants grow on homocysteine and methionine only and, therefore, must be blocked at the cleavage of cystathionine to form homocysteine. Cross-feeding studies subdivided this group with PA-1-553 being fed by PA-1-503 and -506. The placement of PA-1-521 is

questionable due to ambiguous cross-feeding data.

Transduction tests, using the technique of Murphy and Rosenblum (47), confirmed the groupings obtained by growth on intermediates and cross-feeding. The transducing phage used was F116. Data for all phage-sensitive mutants indicated linkage of the methionine loci.

In the first group of mutants, the occurrence of two subgroups would suggest the presence of at least two enzymatic steps. These steps could be: homoserine ⟶ intermediate ⟶ cystathionine with the intermediate being O-succinylhomoserine as in *E. coli* (56, 57), O-acetylhomoserine as in *N. crassa* (57, 58), or a similar compound. Neither the specific defect of each subgroup nor the intermediate has been identified.

In the second group of mutants, cross-feeding and transduction data indicate that the cystathionine to homocysteine reaction may involve more than one enzyme which is coded for by a single locus or closely linked loci.

The biosynthetic pathway for methionine in *P. aeruginosa* is concluded to be quite similar to that in *E. coli*.

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APPENDIX

IDENTIFICATION OF AMINO ACID MUTANTS ISOLATED IN THIS INVESTIGATION

Mutant Number	Amino Acid Requirement
PA-1-85	met
PA-1-86	met
PA-1-87	met
PA-1-88	met
PA-1-89	met
PA-1-90	met
PA-1-91	met
PA-1-92	met
PA-1-93	met
PA-1-94	met
PA-1-95	met
PA-1-96	met
PA-1-97	met
PA-1-98	met
PA-1-99	met
PA-1-502	met, (leu), (ilv)
PA-1-503	met, (ilv)
PA-1-504	met, (ilv)
PA-1-505	leu, (met), (ilv)

Mutant Number	Amino Acid Requirement
PA-1-506	met, (leu), (ilv)
PA-1-509	met, (leu), (ilv)
PA-1-510	met, (leu)
PA-1-512	leu, (met)
PA-1-514	met, (leu), (ilv)
PA-1-516	leu, (met), (ilv)
PA-1-518	met, (leu), (ilv)
PA-1-520	met, (leu), (ilv)
PA-1-521	met
PA-1-524	leu, (met)
PA-1-526	leu, (met), (ilv)
PA-1-528	leu, (met), (ilv)
PA-1-529	leu, (met), (his), (glut), (pro)
PA-1-534	leu, (met)
PA-1-535	ilv
PA-1-537	met, (leu), (ilv), (trp), (tyr)
PA-1-539	leu, (met), (ilv)
PA-1-540	met, (leu)
PA-1-542	leu, (ilv)
PA-1-544	leu, (ilv), (pro)
PA-1-546	leu, (met), (his), (glut), (pro)
PA-1-552	leu, (ilv)
PA-1-553	met

Mutant Number	Amino Acid Requirement
PA-1-555	leu, (met), (ilv)
PA-1-556	leu, (met), (ilv)
PA-1-557	leu, (met), (ilv)
PA-1-558	leu, (met), (ilv)
PA-1-559	leu, (met), (ilv)
PA-1-561	leu, (ilv)
PA-1-562	met, (leu)
PA-1-563	leu, (ilv)
PA-1-565	leu, (met), (ilv)
PA-1-566	met
PA-1-567	leu, (met), (ilv)
PA-1-568	leu, (met), (ilv)
PA-1-570	leu
PA-1-574	leu, (met), (ilv)
PA-1-575	leu, (met), (ilv)
PA-1-576	met, (leu), (ilv), (tyr)
PA-1-577	leu
PA-1-579	leu, (met)
PA-1-580	leu, (met)
PA-1-581	leu, (met)
PA-1-582	leu, (isl)
PA-1-583	leu, (his), (glut), (pro)
PA-1-584	leu, (his)
PA-1-586	leu, (val)

Mutant Number	Amino Acid Requirement
PA-1-587	leu, (val)
PA-1-588	leu
PA-1-590	leu
PA-1-593	leu
PA-1-597	leu, (met)
PA-1-598	leu, (isl)
PA-1-599	leu, (ilv)
PA-1-603	val, (phe), (trp), (tyr)
PA-1-605	leu
PA-1-606	met
PA-1-607	leu
PA-1-608	leu, (met)
PA-1-609	met, (leu)
PA-1-610	leu, (met)
PA-1-611	leu
PA-1-612	leu
PA-1-614	leu, (met)
PA-1-616	leu, (met)
PA-1-619	leu, (met)
PA-1-620	leu, (met)
PA-1-621	leu, (met)
PA-1-622	leu

Abbreviations used: glut, glutamic acid; his, histidine; isl, isoleucine; ilv, isoleucine-valine; leu, leucine;

met, methionine; phe, phenylalanine; pro, proline; trp, tryptophan; tyr, tyrosine; val, valine.

Parentheses indicate amino acids which also allow growth.

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