DISCOVERY AND CHARACTERIZATION OF TWO Tn5 GENERATED pyrA MUTANTS IN *Pseudomonas putida* AND THE GENERATION OF Hfr STRAINS

DISERTATION

Presented to the Graduate Council of the University of North Texas in Partial Fulfillment of the Requirements For the Degree of

DOCTOR OF PHILOSOPHY

By

Laura Gail Liljestrand, B.A., M.S.
Denton, Texas
August 1994
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A *pyrA* mutation in *Pseudomonas putida* was isolated using transposon mutagenesis for the first time. Transposon Tn5 was used to inactivate the *pyrA* gene for carbamoylphosphate synthetase in these mutants. Accordingly, these mutants were defective in pyrimidine and arginine biosynthesis. The suicide vector, pMO75, from *Pseudomonas aeruginosa*, was used to introduce the transposon into the cells. Tn5 was subsequently used to supply homology so that the plasmid pMO75 could be introduced in its entirety into the *Pseudomonas putida* chromosome at the locus of the Tn5 insertion in the *pyrA* gene. Consequently, these strains exhibited high frequency of recombination and were capable of chromosome mobilization. Results of conjugal matings with appropriate recipient strains located the *pyrA* mutation within the auxotrophic region of the *Pseudomonas aeruginosa* chromosome. This *pyrA* mutation was therefore mapped where most marker genes for intermediary metabolism are found. Because of their dual requirement for pyrimidine and arginine, these *pyrA* mutants should prove useful for characterizing the physical and genetic map of *Pseudomonas*. 
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A pyrA mutation in Pseudomonas putida was isolated using transposon mutagenesis for the first time. Transposon Tn5 was used to inactivate the pyrA gene for carbamoylphosphate synthetase in these mutants. Accordingly, these mutants were defective in pyrimidine and arginine biosynthesis. Tn5 was subsequently used to supply homology so that the plasmid pMO75 could be introduced in its entirety into the Pseudomonas putida chromosome at the locus of the Tn5 insertion in the pyrA gene. Consequently, these strains exhibited high frequency of recombination and were capable of chromosome mobilization.

The Bacterial Chromosome: A Short History

It was not always accepted that bacteria have genetic material similar to eukaryotes, due to the lack of its separation from the cytoplasm and the lack of visibility since it does not condense prior to cell division. Before 1940 it was generally thought that changes in bacterial populations may actually be Lamarckian in nature rather than natural selection of spontaneously occurring mutations (Drlica & Riley, 1990). Most genetic work was being done with Drosophila or haploid strains of microorganisms such as Neurospora and Saccharomyces which had the advantages of rapid reproduction and simple
growth conditions. However, microorganisms do not have easily recognizable features except for colony morphology, so it was not until mutations for metabolic defects were studied that real progress was made (Brock, 1988). In 1941, Beadle and Tatum found the first successful mutant cells in *Neurospora crassa* by subjecting cells to UV radiation and then selecting for their descendants that could grow only on supplemented minimal media. The first gene identified was for an enzyme in the biosynthetic pathway for the vitamin, niacin (Watson *et al.*, 1987).

In 1943, one of the first experiments to indicate that the bacterial genetics system was similar to that of eukaryotes measured the frequencies of *Escherchia coli* cells, arising in a culture, that were resistant to the T1 bacteriophage. Fluctuations in frequencies of cells resistant to the bacteriophage, that arose among the different cultures, indicated spontaneously arising mutations (Luria & Delbruck, 1943). Nine years later, spontaneous mutation was demonstrated in *E. coli* when replica plating was developed which allowed single colonies to be rapidly screened for mutants (Lederberg & Lederberg, 1952).

Early studies on *E. coli* and other bacteria required the development of many important techniques. Direct selection utilizes growth conditions of the desired mutant and counterselection kills the parent cells. When it was found in 1946, that *E. coli* carried out genetic recombination, these techniques were used to isolate the recombinant cells and detect the pattern of genes on the bacterial chromosome (Lederberg & Tatum, 1946). By the 1950s, three systems of genetic exchange in bacteria were known, conjugation, transduction and transformation, which would be used to further study the bacterial
chromosome. The discovery of lysogenic phages, some carrying bacterial DNA, that could insert themselves into the bacterial chromosome at fixed points, became a key to genetic mapping. For instance, the transducing phage, lambda, in *E. coli*, made it possible to study the gene for galactose utilization since its close point of insertion to the gene meant when it recombined out, it sometimes picked up the galactose operon (Campbell & Balbinder, 1959).

Studies of conjugation in *E. coli* revealed that bacteria transferred the F+ genes unidirectionally through a point of direct contact from the F+ to the F- cell (Hayes, 1952). If the F+ factor becomes integrated into the chromosome through a crossing-over process, an F+ cell with a high frequency of recombination (Hfr) results. Attachment of an Hfr cell to an F- cell, causes the replication and transfer of donor bacterial genes but, because the actual process may take over an hour, the F+ factor transfer seldom occurs and the recipient remains F- (Wollman, et al. 1956). When it was realized that in a particular Hfr strain, the F+ factor was inserted at a fixed point in the bacterial chromosome because the genes entering the F- cell were always the same, interrupted mating experiments led to the discovery of how close which genes were to the integration site. By 1961, the existence of many Hfr strains with different integration sites made the sequencing of the genes on the *E. coli* chromosome possible (Jacob & Wollman, 1961). Although it was suggested in many studies, it was not until 1963 that it became established that the bacterial chromosome was circular (Cairns, 1963).

During the ensuing years, genetic maps have been constructed for a number of different bacteria, although the most extensive is for the K-12 strain of *E. coli* in which over 1000 genes had been mapped by 1983 (Bachman, 1983).
Different strains have different arrangements of some genes on their chromosomes but most gene locations are highly conserved. This conservation is especially true among related organisms, perhaps because gene position is important in how the gene functions.

New techniques in genetic manipulations were developed when it was found that plasmids and transposons could be engineered to carry foreign DNA. It had long been accepted that genes had fixed locations on the chromosome, thus, Barbara McClintock's work in maize showing the existence of movable genes was largely ignored in the 1940s. But in the 1960s it became clear that *E. coli* also had transposable elements that could randomly insert themselves along the bacterial chromosome knocking out gene function (Shapiro, 1983). Plasmids, small circular dispensable DNA molecules carrying genes for extraneous functions such as antibiotic resistance and conjugation (F+ factor), were found to replicate themselves separately from the main chromosome (Bukhari et al., 1977). The movement of transposons and plasmids carrying genetic markers from one bacterium to another during conjugation became a new tool for mutagenesis and gene mapping.

Recombinant DNA techniques of the early 1970s brought new insight to the genetic maps of the bacterial chromosome. The first restriction nuclease found to cleave at a particular site was located in *H. influenzae* and called *HindIII* (Smith & Wilcox, 1970). This enzyme was used to generate the first restriction map, a map that identified the sites at which the enzyme cut the DNA of the virus SV40 (Danna & Nathans, 1971). Within a few years two powerful DNA sequencing methods were developed and being used. The one by Allan Maxam and Walter Gilbert was based on chemical degradation of the DNA
molecule and the other by Fred Sanger required DNA polymerase to synthesize DNA fragments (Rawn, 1989). By 1975 it had become possible to generate phage and plasmid vectors engineered with restriction sites that allowed the insertion of almost any short piece of DNA. When the plasmid or phage is taken up by bacteria, the inserted DNA is cloned, thus increasing the amount of desired DNA. During the late 1970s, concerned that danger existed in using recombinant DNA, scientists agreed to use cloning only in organisms that could not grow outside a test tube. This hampered this type of experimentation for a few years but, when no danger seemed to exist, an increased number of scientists began investigating the bacterial chromosome (Watson et al., 1983).

During the 1980s, physical maps of the bacterial chromosome were constructed by cutting the DNA with certain restriction endonucleases and then sequencing the nucleotides in specific regions of the gene. As genes are sequenced and overlapping regions located the physical maps generated can be correlated with their relative positions on the genetic map (Kohara, 1990). The genetic map of the bacterial chromosome is invaluable in the study of a bacterium's metabolism and evolution.

**Conjugation**

Joshua Lederberg conceived the idea of crosses between multiple auxotrophs to detect recombination in *E. coli*, proposing to E. L. Tatum that they join forces and use Tatum's biochemical mutants. The classical experiment mixed a culture requiring methionine and biotin with one that required threonine and leucine overnight. Washing and plating the mixed culture on minimal agar yielded genetic recombinants. Each recombinant had incorporated the other
two genes into its chromosome. Transformation was ruled out by adding a sterile filtrate of one culture to that of the other, and observing that no recombinants occurred (Lederberg & Tatum, 1946). However, they interpreted recombination in the light of eukaryotic nuclear fusion and meiotic reduction division with both parent cells contributing equally to the offspring. Over the next four years, as the frequencies of more unselected characters were analyzed, the first genetic map of *E. coli* with twelve characters was published as linear with two branching points at methionine (Stent, 1963).

When William Hayes, working with similar crosses of auxotrophs, killed the Met+ strain (donor of threonine and leucine) with streptomycin, the number of recombinants remained the same. But when he treated the Thr- Leu- Thi- strain (donor of methionine) with streptomycin, no recombination occurred. He interpreted this to mean that transfer of genetic material during conjugation was unidirectional from a donor to a recipient (Hayes, 1952). The Lederbergs, Cavalli and Hayes would continue these crosses for the next few years, adding markers and verifying the sexual polarity in recombination and the existence of a fertility factor F. Hayes surmised that the donor, the F+ cell, only transfers a partial genome to the F- recipient by means of an F factor capable of reproducing itself. Wollman and Jacob coined the term "episome" to characterize an extrachromosomal genetic element that had to be added to the cell externally but could exist in the cell either autonomously or integrated into the chromosome (Wollman et al., 1956).

By the early 1950s both Hayes and Cavalli had discovered strains that could produce recombinants at a frequency a thousand times higher than the original strains. These Hfr strains were apparently donors since they could be
killed by streptomycin without affecting the fertility rate, and the recombinants had mostly the characters of the F- cells. But Hfrs differed from the F+ strains in that they could transfer a limited amount of the F+ genome at a high rate but seldom the F factor itself.

Wollman and Jacob took a different approach to Hfr and F- crosses by studying the number of recombinants that appeared at ten minute time intervals during an hour. A diluted sample was removed from the culture and violently treated in a Waring blender to separate the parent cells before plating on minimal media. They found that certain characters such as threonine and leucine did not appear during the first eight minutes and others such as galactose took even longer to appear. They interpreted this to mean that the transfer of genes was always linear and in the same orientation and, thus, were able to construct the first temporal map of *E. coli* in 1956. When they questioned whether the original F+ strains were actually transferring genetic material at all, they carried out fluctuations tests and replica plating that confirmed that Hfr cells, spontaneously occurring in the F+ strain, were actually responsible for transfer of the donor genome. Study of the order of gene transfer in the different Hfrs led them to postulate that the *E. coli* chromosome was circular (Jacob and Wollman, 1961).

It was about this time that another form of the fertility factor, designated F' or F prime, was discovered by E. H. Adelberg. This form had a preference for a particular site on the F- chromosome and recombinants were actually diploid in nature, having both the Lac- character of the recipient and the Lac+ character of the donor. It was proposed that an F' carried a piece of the Hfr chromosome, obtained by an improper looping out when an Hfr cell reverted to the F+ state.
(Stent & Calender, 1978).

The bridge that forms during conjugation was first shown in a 1957 electron micrograph by T. F. Anderson and more recent photographs show the F pili produced by F\(^+\) cells for attachment to F\(^-\) cells. The role of F pili in conjugation is not well understood but genes for its synthesis are located on the F factor and its attachment to an F\(^-\) cell triggers the replication and transfer of the F factor (Ou and Anderson, 1970). It is thought that DNA is replicated by a rolling circle mechanism and only one strand of the DNA is transferred during conjugation. In the rolling circle mechanism a nick opens one strand of the DNA molecule at the origin. As the free 3'-OH end is extended by DNA polymerase, the newly synthesized strand displaces the original strand which moves to the F- cell. If the F factor is integrated into the main chromosome, the latter will be mobilized during conjugation (Watson et al., 1987).

**Genetic Recombination**

Genetic mapping was based on the premise that the closer genes are linked on the chromosome, the greater the chance they would appear together in the recombinant descendants. Any two donor genes appearing would first have to be transferred and then both would have to be integrated into the recipient chromosome. From Lederberg's simple genetic map of *E. coli* in 1947 to the complex maps of today showing over a thousand genes, all of the maps have remained congruent.

Early theories in bacterial genetic recombination had to be discarded as more became known about molecular genetics. In Hfr crosses with F- strains, a single strand of donor DNA is transferred to the F- cell. The simplest
explanation would be that the donor DNA invades the recipient DNA in a D-loop formation at regions of homology, displacing it. The recombinants are F- recipient cells in which F- genes are lost and the descendants have a recombinant chromosome. During D-loop formation, the existence of heteroduplex DNA intermediaries should exist and detection of these would help substantiate this theory (Stent, 1963).

In 1975, experiments which amplified a plasmid in E. coli yielded figure-of-eight molecules shown to be held together by cross-over. Treatment with a restriction enzyme caused them to resolve into chi shaped structures, the intermediary forms predicted by the Holliday model of recombination (Smith, 1988). In the first step of the Holliday model, DNA duplexes pair and nicks are made in homologous strands. The broken ends can move and each strand crosses over to pair with the complement in the other strand. The nicks are sealed and the crossover point moves by branch migration (Lewin, 1990). Only one pairing would be required in a bacterial cross-over event and the F- genes would be displaced.

Enzymatic processes associated with recombination between Hfr and F- cells are studied with mutations in the recA gene that abolish any recombination and in the recBCD genes which reduce recombination capacity. One model presented proceeds as follows: The RecBCD enzyme attaches to the end of the entering donor DNA and moves along it until it reaches a chi site (5' GCTGGTGG 3') and cleaves the DNA close to this sequence. This strand can now be assimilated into the recipient duplex by the activity of the RecA enzyme which promotes base pairing between the donor strand and complementary bases on the recipient (Smith, 1988).
Plasmids

The term plasmid was first used by Joshua Lederberg to designate a piece of self-replicating extrachromosomal DNA in bacteria. The first one to be discovered and studied was the F factor plasmid for conjugation. Plasmid replicons may have a single-copy control system and replicate once with each cell division or they may have a multicopy control system with the result that there can be several copies in the cell. Each type of plasmid maintains a certain copy number in its bacterial host related to a phenomenon known as plasmid incompatibility. It is thought that a repressor molecule that recognizes the number of origins in a compatibility group may prevent synthesis of more than the copy number (Lewin, 1990). Plasmids usually do not have genes for any essential functions but encode dispensable functions such as antibiotic resistance. Small plasmids are ideal for this since their high copy number can allow gene amplification in bacteria exposed to an antibiotic. But plasmids also can be lost during cell division if the antibiotic is not present in the medium or by gaining resistance to it.

In recombinant DNA technology, plasmids became cloning vectors for manipulating genetic material. The restriction endonucleases that bacteria had evolved to protect themselves from viruses were used to construct chimeric genomes. Stanley Cohen and Herbert Boyer constructed the first of these in 1973 using EcoR1, which recognizes the palindromic sequence of GAATTC and cleaves at either end between GA. Using EcoR1 to cut both a small plasmid with an origin and tetracycline resistance and DNA isolated from an amphibian, they created complementary ends on the two different types of DNA.
Then they mixed toad DNA fragments with the plasmid and allowed bacterial cells to take up the DNA, selecting for cells resistant to tetracycline. The plasmid they had created carried a vertebrate ribosomal RNA gene at the EcoR1 site, a true chimera that never before existed in nature. Since then, insertion of foreign DNA into plasmids and lysogenic viruses, producing recombinant DNA, has become a commonplace tool for the geneticist. The plasmid containing foreign DNA can be introduced into bacteria which then reproduce forming a clone of cells, each of which can be maintained separately, constituting a clone library of the original DNA.

Plasmid cloning vectors with desired improvements have been developed by introducing changes in the replication control systems, placing cleavage sites for certain restriction enzymes in nonessential locations, and adding genes for different antibiotic resistances to naturally occurring plasmids. Transposons also were incorporated into plasmids for vectoring purposes. The plasmids pMO75 and pMO1896 used in this research were engineered to carry R91-5 which has the transposon Tn5 inserted in opposite orientations in the two plasmids.

Transposons

Transposons are DNA sequences that are capable of inserting either themselves or copies of themselves at other chromosomal sites in the absence of homology. They are unable to exist autonomously, replicating only as part of an independent replicon. They can move from a plasmid to a chromosome, from one site to another in the same replicon and be transferred from one cell to another. In size, they range from small insertion sequences of 750 base pairs to
complex transposons of 40,000 bp. Each carries a gene for transposase that allows its insertion and the larger ones carry the resolvase gene and host genes also (Kleckner, 1981).

Transposable genetic elements, first discovered in maize by Barbara McClintock in the 1940s, are found in all major groups of organisms. Transposons in bacteria made their debut in 1968 when insertion sequences were found in the gal operon of E. coli. Analysis of unstable mutants that had appeared revealed DNA sequences of about 1 kb in length that disappeared in revertant organisms. Jacob and Hedges coined the term transposon in 1974 when they postulated that the transfer of the gene for ampicillin resistance from one plasmid to another was being carried by a transposable element (Shapiro, 1983).

The transposable elements of bacteria have been divided into three distinct classes based on genetic organization: Class I includes IS-like modules and composite elements formed from them; Class II includes complex transposons, TnA elements; and Class III bacteriophage Mu. Class I composite transposons can use both a conservative mechanism for transposition in which the sequence is cut out of the host DNA, leaving a lethal gap, and ligated into the cut target DNA, as well as, the replicative method. They can insert more than one copy of the donor replicon in the cell at the time of transposition. Class II complex transposons always use the replicative method of making a copy of the transposon, forming an intermediate co-integrate and using resolvase to separate the donor and recipient. In all cases, a short sequence of 5 to 9 base pairs of host DNA is replicated and joined to each side of the inverted repeats of the transposon when it inserts. The insertion of a
transposon into a gene has the immediate effect of disrupting it and the moving in and out of genes can cause deletions and inversions (Kleckner, 1981).

Transposons might be considered molecular parasites, surviving only by replicating at the expense of other DNA. If transposons are so destructive to the bacterial genome, it is interesting to speculate why transposons are tolerated by bacteria that seem to have evolved a very tight control over their genetic make-up. For instance, genes that are involved in a particular metabolic pathway are controlled by the same promoter of an operon. It is perhaps the existence of transposons that has allowed bacteria to experiment with different genetic arrangements. Too much plasticity in the genome was not good, but the occasional shifting of a gene could have led to the high efficiency of polycistrons. When the gene for transposase came into existence, it may have been an errant piece of DNA moving about destructively, but as it evolved, it would have to regulate itself or disappear with its dead host. Over time, the transposase gene would come to produce only one enzyme molecule or less per generation. Along with phages this enzyme may be one of the causes for bacteria to have evolved methylation of its DNA to protect it. The promoter for transposase is active when DNA is in the hemimethylated state and becomes inactive when the bacterial DNA becomes fully methylated (Kleckner, 1981).

Many of the antibiotic resistance genes on plasmids are enclosed in transposons, a decided advantage since plasmids can be lost or selected against. Since transposons can recombine without homology, multiple resistance to antibiotics in bacteria can arise when different transposons insert into the same plasmid. The advantages of having transposons seem to far outweigh their apparent destructiveness.
Figure 1. Transposition Models (from Watson et al., 1987)

a) Transposase binds both transposon ends and also the new target site.
b) Specific cuts are made in both DNAs, and they are rejoined after strands are exchanged.
c) In Simple Transposition, the transposon is then cut away from the host DNA, and
d) target site duplications are completed by DNA polymerase.
e) In Replicative Transposition, each transposon strand is copied by DNA polymerase,
f) yielding a cointegrate
g) that is separated by resolvase.
Old target site duplication

Transposon
Original host DNA

Transposase makes a staggered cut at target site and cuts each transposon end.

Arrows show where strands are reconnected.

DNA polymerase completes the target site duplication.

Repair synthesis

New synthesis by DNA polymerase

Resolution site

Resolvase divides the co-integrate by recombination between sites on the two transposon copies.
Tn5 Transposition

The most widely used transposon for mutagenesis and the one used in this research is Tn5. It is a Class I composite transposon, 5.7 kb in length, its target site duplication is 9 base pairs and it has IS 50. Its genetic marker is kanamycin resistance. Tn5 appears to regulate its own activity by specifying a diffusible negative regulator that suppresses the activity of a Tn5 element resident in the cell and any newly introduced Tn5 element. Thus, Tn5 transposition is more frequent when it first enters a cell not hosting it. The flanking element, IS50-Right, is fully functional for transposition but IS50-Left is completely defective. The latter has a single base change resulting in a stop codon that causes premature termination of the encoded proteins thus eliminating redundant transposition. The promoter for the kanamycin resistance gene is within IS50-Left and the gene's coding region begins just to its right. All essential sites occur within 100 base pairs from the end which contains a greater concentration of A-T pairing than does the center. The terminal inverted repeat sequences of IS50-Right must contribute important information because it must be intact if transposition is to occur. It probably contains the nucleotide sequences recognized by transposase for precise excision. Symmetry at the ends of all transposons may be functional as well as structural since both direct-repeat and inverted-repeat sequences transpose, thus enabling DNA replication at the target site to begin correctly at either end of the element (Kleckner, 1981).

As a Class I composite transposon, Tn5 can transpose by both the simple conservative method and by replication, although the former is thought to occur over a hundred times more often. A transposition model is shown in Figure 1.
Transposase (the gene for which is located in IS50-Right of Tn5) is newly synthesized and binds both transposon ends, as well as, the new DNA target site. It makes staggered cuts at each of the three sites, at the end of the inverted repeats on the transposon and at a random point on the target DNA. The strands of the target DNA are then connected to that of the transposon. In the simple conservative method, a second set of cuts releases the old replicon leaving a lethal gap, and DNA polymerase completes a 9 base pair sequence duplication of the target DNA on either end of the transposon insertion. In the more involved replicative transposition, DNA polymerase synthesizes the entire transposon and completes the 9 base pair duplication. For a short time this connects the old and new replicons into a cointegrate, which then separates, leaving a copy at the old site. Class II transposons can produce the enzyme resolvase for the cointegrate separation (Watson et al., 1987).

Tn5 was first identified in *E. coli* but has subsequently been transferred to a wide variety of Gram-negative organisms including pseudomonads. It has a high frequency of transposition and a low specificity of insertion, ideal properties for transposon mutagenesis (Holloway, 1986).

**Gene Transfer in Pseudomonas**

The three main types of gene transfer, transformation, transduction and conjugation, are known to occur in *Pseudomonas*. Gene transfer by transduction has been an important procedure for gene mapping in both *P. aeruginosa* and *P. putida*. Transduction occurs naturally among bacteria, when viruses mistakenly incorporate bacterial DNA into themselves and so transfer it into other cells they infect. Generalized transducing phages, such as
F116 and G101, can mediate transfer of genes from both chromosomal and plasmid replicons. Although all chromosomal genes seem equally accessible to transduction, genes derived from plasmids are not. Of the two major classes of transferable plasmids, the FP and RP factors, the RP factors are readily transducible but the FP factors have never been found (Stanisich & Richmond, 1975).

Studies of gene transfer by conjugation in *P. aeruginosa* and *P. putida* usually use the transferable plasmids, FP or RP types. The FP factors have not been associated with antibiotic resistance but several do confer resistance to mercuric ions or UV radiation (Pemberton & Clark, 1973). In 1955, the FP sex factor was originally detected in *P. aeruginosa* PAT when recombinants were discovered under conditions where cell-to-cell contact had to take place for gene transfer (Holloway, 1955). The RP factors were detected because of the resistance they confer to a variety of antibiotics, in particular, carbenicillin. They have come into wide use because they can mediate their own transfer by conjugation and sometimes mobilize chromosomal genes (Stanisich & Richmond, 1975). Many of the naturally occurring RP plasmids have been engineered to carry transposons which effect chromosome mobilization in a number of gram negative organisms. The IncP-1 plasmid, R68.45, harbors IS21 which may be responsible for the ability of the plasmid to mobilize the chromosome at a low frequency and from multiple regions (Pischal & Farrand, 1983).

While the IncP-1 plasmids are used for mapping in *P. aeruginosa* they are not always suitable for other species of *Pseudomonas*. However, the Inc-10 plasmid, R91-5, has been found to be highly effective in *P. putida*. The
replication region of the R91-5 plasmid suggests that it does not function in a manner analogous to other well characterized plasmids (Davies & Krishnapillai, 1990). Although the R91-5 plasmid can transfer to and integrate into the chromosome of other *Pseudomonas* species, it can only replicate in *P. aeruginosa* (Holloway, 1975). In this research, the R91-5 plasmid (pM075) of the donor, *Pseudomonas aeruginosa* PAO 11, is transferred during conjugation with high efficiency to the recipient, *P. putida* PRS2000.

*Pseudomonas* Characteristics and Importance

In 1884, just one hundred years ago, W. Migula first described the genus *Pseudomonas*. For 70 years, many poorly characterized species were assigned to this genus by workers ignoring all attempts to set up a rational classification of the *Pseudomonas* species. In the 1960s, work based on examination of phenotypic characteristics and nutrition, a subdivision of the genus into species and species groups was finally established. Important diagnostic properties were used that include the colony structure, the odor, the production of characteristic pigments, ideal temperature growth, denitrification, gelatin liquefication and the ability to utilize certain carbon compounds (Palleroni, 1984). The cells of *Pseudomonas* are Gram negative and are usually straight rods from 1 to 4 micrometers in length with active swimming motility due to polar flagella. All members are aerobic chemoorganotrophs that do not form endospores, stalks or sheaths. The taxonomy of this genus places *P. aeruginosa* and *P. putida* in the fluorescent species of Group I based on rRNA homology. Some important characters for the diagnosis of *P. aeruginosa* are the copper rust color of the colony with shining metallic patches, the fruity
smell, and the fast growth at 37°C. It is an absolute aerobe except in media containing nitrate where reduction to nitrogen gas becomes visible. *P. putida* can be identified by its being unable to liquefy gelatin or carry out denitrification or grow well above 35°C (Palleroni, 1975).

*Pseudomonas* has always had research prominence in microbiology because of its role in human infection as an opportunistic pathogen and because of the wide range of its biochemical abilities. In recent years, pseudomonads have attracted the attention of phytopathologists which study the species that act as specific biopesticides by inhibiting the growth of root pathogens. Other pseudomonads have been identified as detoxifying chemical wastes (Holloway, 1992).

**The *Pseudomonas* Genome**

Genetic knowledge of the pseudomonads is somewhat limited in comparison to *E. coli*, however, recent advances in molecular biological techniques such as transposon mutagenesis and cloning are providing new information on the *Pseudomonas* genome. Extensive chromosomal maps are important in establishing the circularity of the chromosome, for the understanding of the order of the genes, or if the genes are on the chromosome or a plasmid. Comparison of genomes of different species gives evidence as to how such genomes may have evolved. Genetic systems have been developed for mapping both *P. aeruginosa* PAO and *P. putida* PPN (Holloway & Morgan, 1989).

The development of the many techniques to isolate and manipulate DNA has led to the construction of a genetic map for *P. aeruginosa* with over 300
markers and a genome size of 5,862 kb, one of the largest among prokaryotes. The genome sizes of many of the human and animal pathogens range from 900 to 2,650 kb and the genome sizes of *E. coli* K12 and *Bacillus subtilis* are about 4,700 kb. One reason for the larger genome size of *P. aeruginosa* is that, unlike pathogens that rely on cellular hosts, *P. aeruginosa* has an extensive number of catabolic genes to enable it to be a free-living bacterium that is nutritionally independent (Ratnaningsih *et al.*, 1990). A comparison of the gene arrangement and similar genome size of *P. aeruginosa* PAO with that of *P. putida* PPN has led to proposals that their evolution is closely related. One important difference, however, is an auxotroph-rich region of 3,300 kb in length for *P. aeruginosa* but only 2,600 kb for *P. putida*. In *P. putida* PPN, 84% of the known auxotrophic markers are found in 38% of the genetic map, but in *P. aeruginosa*, 79% of the auxotrophic markers are found in 48% of the genetic map (Holloway & Morgan, 1986). *P. aeruginosa* has virulence determinants, lacking in *P. putida*, which indicates that it has become an opportunistic pathogen only recently (Ratnaningsih *et al.*, 1990). Modern day pseudomonads may have acquired new genes and new metabolic functions by integrating plasmid fragments into an earlier smaller chromosome. Such accretion of new genes probably occurred in regions other than the essential-function region (Holloway *et al.*, 1990). *P. putida* MW was found to carry 56 kb of DNA almost identical with a part of the TOL plasmid integrated into its chromosome. The non-random nature of the TOL insertions into the PAO chromosome indicate that there is a preferred target for the transposable elements (Sinclair & Holloway, 1991).

Some of the methods by which the chromosome map for *P. putida* PPN
have been constructed are listed in Figure 2. Note that pyrB was one of those mapped by Tn5 mutagenesis and the generation of Hfr donors mobilizing the chromosome from the site of insertion (Holloway et al., 1990). My research has generated two pyrA mutants in P. putida by Tn5 mutagenesis with both arginine and uracil requirements and P. putida Hfr donor strains that can mobilize the chromosome from the site of the pyrA insertion in either direction.

**Generation of Pseudomonas Hfr Donor Strains**

Chromosome mobilizing plasmids and the ability to insert transposons into the Pseudomonas genome have become useful tools in its mapping. In P. aeruginosa, an IncP1 plasmid loaded with a transposon has been used to construct a wide range of Hfr donors with different origins that provide mapping access to the entire chromosome (O'Hoy & Krishnapillai, 1987). The conjugative plasmid R68.45 mobilizes the P. aeruginosa PAO chromosome from multiple sites located in different chromosome regions. Interrupted mating experiments with R68.45 indicate that the plasmid and FP2 appear to promote chromosome transfer at similar rates, but unlike FP2, the transfer is non-polarized (Haas & Hollaway, 1978).

To obtain Hfrs for P. putida, the IncP-10 plasmid R91-5 is highly effective in the fluorescent pseudomonads because there is a high conjugal efficiency between P. aeruginosa and other Pseudomonas species (Holloway, 1975). Although the plasmid R91-5 can only replicate in P. aeruginosa, it will transfer by conjugation from P. aeruginosa to P. putida with high frequency and integrate into the bacterial chromosome creating an Hfr donor. A wide range of Hfrs with known chromosomal transfer origins have been constructed by first
Figure 2. Chromosome Map of *Pseudomonas putida* PPN (from Holloway *et al.*, 1990). The markers with an allele number commencing with an 8 were generated by Tn5 mutagenesis, and in each case, an Hfr donor mobilizing the chromosome from the site of insertion was used in mapping.
Figure 2. Chromosome map of P. putida PPN (ATCC 12633). Markers joined to the map by means of a bar have been mapped by interrupted mating. Those with an allele number commencing with an 8 were generated by Tn3 mutagenesis, and in each case Hfr donors mobilizing the chromosome from the site of insertion have been generated and used in mapping. Markers in parentheses have been detected on R-plasmid plasmids by means of interspecific complementation but have not yet been identified by mutation. Other markers have been mapped by means of P1Hmph-mediated transduction. Marker designations: arg, arginine requirement; ara, aromatic amino acid biosynthesis; att, benzoate utilization; cat, catechol utilization; cy, cysteine requirement; glu, glucose and glutamate uptake; his, histidine requirement; lnu, histidine utilization; lru, requirement for isoleucine plus valine; leu, leucine requirement; ly, lysine requirement; mdc, mandelate utilization; met, methionine requirement; nal, nalidixic acid resistance; pac, p-aminocinchoninic acid utilization; pat, phenylacetate utilization; pol, phenylalanine utilization; pca, protocatechuate utilization; phe, phenylalanine utilization; phe, p-hydroxybenzoate utilization; pro, proline requirement; pyr, pyrimidine requirement; pur, purine requirement; rec, recombination deficient; rif, rifampicin resistance; ser, serine requirement; streptomycin resistance; thr, threonine requirement; trp, tryptophan requirement; val, valine requirement; val, requirement for valine plus isoleucine plus leucine.
infecting \textit{P. putida} with the Tn5 transposon and then reintroducing R91-5 loaded with Tn5. A homologous recombination event occurs between the plasmid Tn5 and the Tn5 in the chromosome. This produces an Hfr with the mobilization origin at the same site as the original Tn5 insertion (Holloway et al., 1990). The same transposon is inserted in opposite orientation in the plasmids R18 and R91-5, thus, each chromosomal insertion site of the transposon could act as an origin site in either direction, depending on the plasmid used. Genes on either side of the insertion site can thus be mapped (Holloway, 1986).

A recent study has shown the plasmid pMO75 to be a suitable vector for chromosomal mutagenesis of \textit{P. putida} PPN. Most of the 62 auxotrophs in the study yielded some prototrophic revertants which were Km sensitive, suggesting that the mutations resulted from a single Tn5 insertion, which was lost from the revertant. The reintroduction of pMO75 into the auxotrophs by plate mating with PA011, produced Hfrs, 95% of which were able to act as donors in replica-plate matings. Each Tn5 mutant yielded two classes of Hfr donor, transferring chromosome in opposite directions, but with one direction favored 8:1. This suggests that pMO75 can integrate in either orientation, mediated by homologous recombination between Tn5 on the chromosome with Tn5 on the plasmid, aligned in the same direction. The 1.5 kb inverted terminal repeats of Tn5 make it possible to orient in either direction. The high frequency of proximal marker transfer located the approximate position of the transfer origins by spot matings with recipient strains carrying auxotrophic markers of known map position. Five previously unidentified markers were mapped by means of interrupted matings. The size of the \textit{P. putida} genetic map was revised from 103 min to 88 min (Strom et al., 1990). My research was based on
this study and others like it except that my study screened for a mutant with a
defective pyrimidine pathway.

Pyrimidine Biosynthesis

Purines and pyrimidines are normally not present in the cell as free
bases or nucleosides but are found almost exclusively as nucleotides. In
addition to ATP and GTP which drive many biosynthetic processes, the
nucleotides are the immediate precursors of nucleic acids and certain
coenzymes. With the exception of CTP, which is formed by amination of UTP,
the ribonucleoside triphosphates are derived \textit{de novo} from corresponding
ribonucleoside monophosphates. The latter may be synthesized \textit{de novo} from
simpler precursors or derived from salvage pathways but, in either case, ribose
5-phosphate comes from 5-phosphoribosyl-1-pyrophosphate (PRPP) (Neuhard
and Nygaard, 1987).

The six enzymes for the first six reactions of \textit{de novo} pyrimidine
biosynthesis given in Figure 3 are coded for by the six unlinked loci, \textit{pyrA}
through \textit{pyrF}. There is no evidence that these \textit{pyr} genes constitute a regulon
(Neuhard, 1985). A Tn5 insertion into any of these loci should interfere with the
production of both UTP and CTP.

\textit{pyrA} mutations result in different phenotypes in different organisms. In
\textit{Neurospora} there are two distinct classes of carbamoylphosphate synthetases,
one specific for arginine biosynthesis and the other for pyrimidine biosynthesis.
A mutant missing the enzyme in one pathway will make carbamoylphosphate
available from the other pathway, eliminating an arginine or uracil requirement.
In \textit{E. coli} and \textit{S. typhimurium}, a \textit{pyrA} mutant requires both arginine and uracil
Figure 3. *De Novo* Biosynthesis of Pyrimidines (information from Rawn, 1989) Carbamoylphosphate synthetase, in the first reaction, also functions in the biosynthesis of arginine. This is the enzyme missing in the *pyrA* mutants discovered in this research.
pyrA carbamoylphosphate synthetase
1) Glutamine + ATP + HCO₃⁻ → Carbamoylphosphate + ADP + glutamate

pyrB aspartate transcarbamoylase
2) Carbamoylphosphate + aspartate → Carbamoylaspartate + Pi

pyrC dihydroorotase
3) Carbamoylaspartate → Dihydroorotate + H₂O

pyrD dihydroorotate dehydrogenase
4) Dihydroorotate + NAD⁺ → Orotate + NADH + H⁺

pyrE orotate phosphoribosyltransferase
5) Orotate + PRPP → Orotidine 5' monophosphate + PPi

pyrF orotidylate decarboxylase
6) Orotidine 5' PO₄ + H₂O → Uridine 5' monophosphate + CO₂

pyrH uridylate kinase
7) Uridine 5' monophosphate + ATP → UDP + ADP

ndk nucleoside diphosphate kinase
8) UDP + ATP → UTP + ADP

pyrG CTP synthetase
9) UTP + glutamine + H₂O + ATP → CTP + glutamate + ADP + Pi
in the medium. (O'Donovan & Neuhard 1970). In *Pseudomonas*, the latter appears to be true also. Figure 4 shows that there is a carbamyl phosphate requirement to convert ornithine to citrulline in arginine biosynthesis.

The utilization of preformed pyrimidine compounds by the salvage pathways allows further characterization of mutants. Uracil is converted to UMP by uracil phosphoribosyltransferase and pyrimidine starvation results in increased levels of the enzyme. Cytosine can only be metabolized through deamination to uracil by the enzyme cytosine deaminase. Cytidine utilization is deamination by cytidine deaminase. Uridine is converted to UMP by either phosphorylation catalyzed by uridine kinase or by phosphorolytic cleavage to uracil and ribose 1-phosphate which are then converted to UMP by uracil phosphoribosyltransferase. (Neuhard & Nygaard, 1987).

Because of their dual requirement for pyrimidine and arginine, the *pyrA* mutants discovered in this research should prove useful for characterizing the physical and genetic map of *Pseudomonas*. 
Figure 4. The Biosynthesis of Arginine (from Rawn, 1989). The enzyme, ornithine transcarbamylase, governs the removal of the phosphate from carbamyl phosphate and the addition of CO-NH$_3$ to ornithine to form citrulline. Since carbamyl phosphate cannot be produced in a *pyrA* mutant, this is the step that is interrupted in synthesizing arginine.
Carbamyl phosphate → Citrulline → Carbamyl phosphate → Argininosuccinate → Aspartate → ATP → AMP, PP, → Fumarate → Argininosuccinate → Arginine
CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains for Mutagenesis

The donors were both *Pseudomonas aeruginosa* PAO11. The genotype is *trpCD54, nal-19, and ton-1* and the phenotype is Trp- with resistance to carbenicillin (Cb) and kanamycin (Km). Donor PAO11 pMO75 has the plasmid R91-5::Tn5 inserted. The plasmid R91-5 is *tra* + and Cb resistant and the transposon Tn5 is Km resistant. Donor PAO11 pMO1896 has the plasmid in reverse orientation of pMO75. *P. aeruginosa* was grown in LB medium with 200ug Km per ml added to maintain the plasmid and was incubated at 37°C. The source was Bruce Holloway, Monash University, Victoria, Australia.

The recipient was *Pseudomonas putida* PRS 2000 (Roger Stanier). This strain is wild type, sensitive to Km and naturally resistant to Cb. It was grown on *Pseudomonas* minimal medium with succinate and incubated at 30°C. The source was L. N. Omston, Yale University, New Haven, CT.

Bacterial Strains for Mapping *pyrA*

The bacterial strains were all auxotrophs of *Pseudomonas aeruginosa* from Bruce Holloway, Monash University, Victoria, Australia. Table 1 lists the 6 different bacterial strains used in mating with the Hfrs. The genetic markers were checked by growing each strain on *Pseudomonas* minimal medium that...
was supplemented with the appropriate amino acids or purines listed in Table 3. Table 2 lists each PAO strain, the supplements added to each plate, and the presence or lack of growth on that plate. If the strain grew on minimal medium without the supplement for a particular marker, that marker was not used in mating with the Hfrs because the mutation had been lost.

Solutions

Stock solutions of uracil, arginine, cytosine, cytidine, uridine, orotate, citrulline, and all the supplements in Table 3 were maintained in concentrations of 2 mg per ml. The purines and pyrimidines were dissolved at pH 8.0 and the rest at pH 7. The antibiotics, kanamycin monosulfate and an ampicillin sodium salt, were kept frozen in a 100 mg per ml concentration. All mention of kanamycin in any procedure is for a concentration of 200 ug per ml, and for ampicillin, a concentration of 1 mg per ml.

Media for All Procedures

The *Pseudomonas* minimal medium plates for *P. putida* PRS 2000, both the wild type and the pyrA mutants were made as follows: 25 ml 0.5 M KH$_2$PO$_4$, 25 ml 0.5 M Na$_2$HPO$_4$, 10 ml 10% (NH$_4$)$_2$SO$_4$ and 10 ml concentrated base were added to 430 ml distilled water and sterilized separately from 15 g purified agar (Sigma) dissolved in 500 ml distilled water. At 45°C 10 ml 1M succinate and any supplements were added to the salts and the mixture was combined with the agar. 40 ug arginine per ml, 40 ug uracil per ml, and 200 ug kanamycin per ml were added to the minimal medium for
the pyrA mutants. The Hfrs were also grown on the supplemented medium plus 1 mg ampicillin per ml.

Standard LB agar plates and broth were used for all PAO strains since they were all auxotrophs. LB medium was prepared as follows: 8.0 g bacto-tryptone, 5.0 g yeast extract and 5.0 g NaCl were added to distilled water to a volume of one liter. The PAO11 strains and the Hfrs required 200 µg kanamycin per ml to retain the plasmid.

Standard YT plates were used for the mating procedure. YT medium was prepared as follows: 10.0 g bacto-tryptone, 5.0 g yeast extract, 10.0 g NaCl, and 18 g agar were added to distilled water to a volume of one liter.

King's Medium B agar was prepared using 20 g agar, 20 g proteose peptone No.3, 1.5 g K₂HPO₄, anhydrous, 1.5 g MgSO₄·7H₂O, and 15 ml glycerol, brought to a one liter volume with distilled water.

Mating and Screening Procedures

The mating and screening procedures were adapted from experimentation developed by Bruce Holloway (Strom et al., 1990). Since they are not standard procedures but had to be developed, they are described in the RESULTS section.

Replica Plating

The first method attempted, that of using velvet cloth to lift the colonies from the screening plates to the replica plates, was unsuccessful. All patching was subsequently done by toothpicks, by removing a colony from the screening
plate, first to the master plate and then to the selection plates that were gridded to match.

Growth Curves for pyrA Mutants

To compare growth curves on the mutants grown in uracil, cytosine, cytidine, and uridine, the procedure was set up as follows. Each mutant was grown up overnight in *Pseudomonas* minimal broth plus succinate, uracil, arginine, and kanamycin on a different day. They were spun down to form pellets which were washed in 0.85% saline. They were spun down again and the pellets were resuspended in 3 ml of saline. Growth flasks for the Klett spectrophotometer were inoculated with 0.5 ml of the mutant in 50 ml of growth media. Each flask had *Pseudomonas* minimal broth plus succinate, arginine, kanamycin and 40 ug of either uracil, cytosine, uridine or cytidine per ml. The flasks were shaken in the 30°C incubator and readings taken every half hour for approximately 6 hours. This procedure was run several times on both mutants.

Procedure to Locate pyrA Mutants Without the Transposon

In an attempt to create true mutants that no longer had the transposon, the pyrA mutants were grown overnight for five successive days without kanamycin to move the transposon out of the pyrA region leaving a mutation there. The procedure developed is described in the RESULTS section.

Procedures for Hfr Generation

The two pyrA mutants were reinfected with the R91-5 :: Tn5
plasmid to generate Hfrs. The mating and screening procedures are described in the RESULTS section.

Procedures to Mate Hfrs with PAO Strains

Four *P. putida* Hfr donors (two mutant G1 and two mutant G2) and each of the six *P. aeruginosa* PAO recipient strains were grown up overnight in LB broth. The mating procedure described earlier that washed the donors and recipients free of any residual broth was used. *Pseudomonas* minimal medium plates with succinate and kanamycin were spread with the supplements required for recipient growth, lacking the one supplement that was being tested. The supplements necessary for each strain are listed in Table 3. In the control plates listed in Table 3, the supplements also were added to a set of minimal medium plates that did not have kanamycin. The significance of these controls and the other controls and tests that were done are discussed in RESULTS and DISCUSSION. Counterselection for the donor Hfr was the lack of uracil and arginine, and for the recipient PAO strain, the missing supplement.
Table 1. Auxotrophic Mutations of *Pseudomonas aeruginosa* and Their Chromosomal Location

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic marker</th>
<th>Map position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO18</td>
<td><em>pur</em>-66</td>
<td>50'</td>
</tr>
<tr>
<td></td>
<td><em>pro</em> B64</td>
<td>4'</td>
</tr>
<tr>
<td>PAO 222</td>
<td><em>met</em>-28</td>
<td>32'</td>
</tr>
<tr>
<td></td>
<td><em>trpC6</em></td>
<td>36'</td>
</tr>
<tr>
<td></td>
<td><em>lys</em>-12</td>
<td>20'</td>
</tr>
<tr>
<td></td>
<td><em>his</em>-II4</td>
<td>17'</td>
</tr>
<tr>
<td></td>
<td><em>proA82</em></td>
<td>40'</td>
</tr>
<tr>
<td></td>
<td><em>ilv B/C226</em></td>
<td>7'</td>
</tr>
<tr>
<td>PAO 944</td>
<td><em>pur</em>-67</td>
<td>89'</td>
</tr>
<tr>
<td></td>
<td><em>hr</em>-48</td>
<td>42'</td>
</tr>
<tr>
<td></td>
<td><em>cys</em>-54</td>
<td>79'</td>
</tr>
<tr>
<td>PAO 12</td>
<td><em>pur</em>-136</td>
<td>25'</td>
</tr>
<tr>
<td></td>
<td><em>leu</em>-8</td>
<td>42'</td>
</tr>
<tr>
<td>PAO 949</td>
<td><em>pur</em>-67</td>
<td>89'</td>
</tr>
<tr>
<td></td>
<td><em>thr 9001</em></td>
<td>42'</td>
</tr>
<tr>
<td></td>
<td><em>cys</em>-59</td>
<td>94'</td>
</tr>
<tr>
<td>PAO 392</td>
<td><em>met</em>-28</td>
<td>32'</td>
</tr>
<tr>
<td></td>
<td><em>rp C6</em></td>
<td>36'</td>
</tr>
<tr>
<td></td>
<td><em>lys</em>-12</td>
<td>20'</td>
</tr>
<tr>
<td></td>
<td><em>pro A82</em></td>
<td>40'</td>
</tr>
<tr>
<td></td>
<td><em>ilv B/C 226</em></td>
<td>7'</td>
</tr>
<tr>
<td></td>
<td><em>his</em>-158</td>
<td>1'</td>
</tr>
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Table 2. Growth of *P. Aeruginosa* Auxotrophs

<table>
<thead>
<tr>
<th>Min med plus:</th>
<th><em>no growth should occur</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAO 18:</strong></td>
<td></td>
</tr>
<tr>
<td>1) ade, pro</td>
<td>growth</td>
</tr>
<tr>
<td>2) adenine</td>
<td>no growth</td>
</tr>
<tr>
<td>3) proline (no ade)</td>
<td>*growth</td>
</tr>
<tr>
<td><strong>PAO 222:</strong></td>
<td></td>
</tr>
<tr>
<td>1) met, trp, lys, his, pro, ilv</td>
<td>growth</td>
</tr>
<tr>
<td>2) trp, lys, his, pro, ilv</td>
<td>no growth</td>
</tr>
<tr>
<td>3) met, lys, his, pro, ilv</td>
<td>no growth</td>
</tr>
<tr>
<td>4) met, trp, his, pro, ilv</td>
<td>no growth</td>
</tr>
<tr>
<td>5) met, trp, lys, pro, ilv</td>
<td>no growth</td>
</tr>
<tr>
<td>6) met, trp, lys, his ilv</td>
<td>no growth</td>
</tr>
<tr>
<td>7) met, trp, lys, his, pro</td>
<td>no growth</td>
</tr>
<tr>
<td><strong>PAO 944:</strong></td>
<td></td>
</tr>
<tr>
<td>1) ade, thr, cys</td>
<td>growth</td>
</tr>
<tr>
<td>2) ade, thr</td>
<td>no growth</td>
</tr>
<tr>
<td>3) ade, cys</td>
<td>no growth</td>
</tr>
<tr>
<td>4) thr, cys (no ade)</td>
<td>*growth</td>
</tr>
<tr>
<td><strong>PAO 949:</strong></td>
<td></td>
</tr>
<tr>
<td>1) ade, thr, cys</td>
<td>growth</td>
</tr>
<tr>
<td>2) ade, thr</td>
<td>no growth</td>
</tr>
<tr>
<td>3) ade, cys</td>
<td>no growth</td>
</tr>
<tr>
<td>4) thr, cys</td>
<td>no growth</td>
</tr>
<tr>
<td><strong>PAO 12:</strong></td>
<td></td>
</tr>
<tr>
<td>1) ade, leu</td>
<td>growth</td>
</tr>
<tr>
<td>2) ade</td>
<td>no growth</td>
</tr>
<tr>
<td>3) leu</td>
<td>no growth</td>
</tr>
<tr>
<td><strong>PAO 392:</strong></td>
<td></td>
</tr>
<tr>
<td>1) met, trp, lys, pro, ilv, his</td>
<td>growth</td>
</tr>
<tr>
<td>2) trp, lys, pro, ilv, his</td>
<td>no growth</td>
</tr>
<tr>
<td>3) met, lys, pro, ilv, his</td>
<td>no growth</td>
</tr>
<tr>
<td>4) met, trp, pro, ilv, his</td>
<td>no growth</td>
</tr>
<tr>
<td>5) met, trp, lys, ilv, his</td>
<td>no growth</td>
</tr>
<tr>
<td>6) met, trp, lys, pro, his</td>
<td>no growth</td>
</tr>
<tr>
<td>7) met, trp, lys, pro, ilv</td>
<td>no growth</td>
</tr>
</tbody>
</table>
Table 3. Supplements for *P. aeruginosa* Auxotrophs

The supplements were added to *Pseudomonas* minimal medium plus succinate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>1)</th>
<th>2)</th>
<th>3)</th>
<th>4)</th>
<th>5)</th>
<th>6)</th>
<th>7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO 18:</td>
<td>ade, pro (control)</td>
<td>ade</td>
<td>pro</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO 222:</td>
<td>met, trp, lys, his, pro, ilv (control)</td>
<td>trp, lys, his, pro, ilv</td>
<td>met, lys, his, pro, ilv</td>
<td>met, trp, his, pro, ilv</td>
<td>met, trp, lys, pro, ilv</td>
<td>met, trp, lys, his, pro</td>
<td></td>
</tr>
<tr>
<td>PAO 944:</td>
<td>ade, thr, cys (control)</td>
<td>ade</td>
<td>thr</td>
<td>cys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO 949:</td>
<td>ade, thr, cys (control)</td>
<td>ade</td>
<td>thr</td>
<td>cys</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO 12:</td>
<td>ade, leu (control)</td>
<td>ade</td>
<td>leu</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PAO 392:</td>
<td>met, trp, lys, pro, ilv, his (control)</td>
<td>trp, lys, pro, ilv, his</td>
<td>met, lys, pro, ilv, his</td>
<td>met, trp, pro, ilv, his</td>
<td>met, trp, lys, ilv, his</td>
<td>met, trp, lys, pro, ilv</td>
<td></td>
</tr>
</tbody>
</table>
This research was performed in three sections:

I. Developing mating and screening procedures to search for, discover and then characterize two pyrA mutants in *Pseudomonas putida*

II. Generating *P. putida* Hfrs from the pyrA mutants

III. Mating the pyrA Hfrs to auxotrophs of *P. aeruginosa* to locate the relative position of the pyrA gene on the genetic map.

I. Discovery of pyrA *P. putida* Mutants

Mating and Screening

The procedure to locate pyrimidine mutants in *Pseudomonas putida* PRS20000 was developed as follows. The donor, *P. aeruginosa* PAO11, was grown overnight at 37°C in LB broth with 200 ug kanamycin per ml to ensure the retention of the plasmid. The recipient *P. putida* PRS2000 was grown overnight at 30°C in LB broth without kanamycin. The next day, both donor and
recipient cells were centrifuged to obtain pellets that were washed with 0.85% sterile saline and centrifuged again. The final pellets were resuspended in one ml of saline. The mating of the donor and recipient cells was done on YT plates without kanamycin because it requires some time for the kanamycin resistance gene to be transferred. Samples of 100 ul of donor and recipient were spread together on YT plates and incubated at 30°C for four hours. Three ml of saline were added to each mating plate and the cells were washed off with a glass spreader. The cell suspensions on each plate were pipetted with a Pasteur pipette to different sterile tubes and mixed on a vortex mixer. Samples of 100 ul of the mating mixture from each tube were spread on a screening plate of *Pseudomonas* minimal medium with succinate and kanamycin and appropriate markers added. These plates were incubated at 30°C for 48 hours.

Counterselection against the parents was as follows. The donor strain, *P. aeruginosa* PAO 11, is a Trp- mutant unable to grow on the minimal medium screening plate. The recipient strain, *P. putida* PRS2000, is sensitive to the 200 ug per ml concentration of kanamycin. These sensitivities and requirements were confirmed before this procedure was begun. The original donor and recipient cells were also transferred to the same screening plate at the same time as controls and checked for lack of growth. There was no growth on any of the control plates.

To increase the chances of locating mutants, each time a mating procedure was run, 10 mating plates were done. A minimum of two screening plates were generated from each of the mating plates, thus, 20 plates of colonies were screened during each procedure. Approximately two to five sets of replica plates, each with 50 patched colonies, were taken from each of the
screening plates. Thus, a minimum of 40 sets of replica plates were generated and approximately 2000 colonies observed.

The purpose of the screening was to locate *Pseudomonas putida* mutants that were unable to synthesize uracil. The first screenings were done on *Pseudomonas* minimal medium with succinate, uracil and kanamycin. The kanamycin ensures that the recipient *P. putida* PRS2000 cells being screened have received the transposon from the donor *P. aeruginosa* PAO 11. The minimal plates with uracil added allows *P. putida* mutants which have the transposon located in a gene in the pyrimidine pathway to survive but eliminates many other auxotrophs. The second screenings were done on *Pseudomonas* minimal medium with succinate, uracil, arginine and kanamycin. The addition of arginine increased the survival chances of the mutants as explained in the DISCUSSION.

Replica plating was done with sterile toothpicks, removing a colony from the screening plate, first to the master plate and then to the selection plates that were gridded to match. The colonies on the screening plates during the first screenings were patched to two types of *Pseudomonas* minimal plates with succinate and kanamycin, the master plate with uracil and a selection plate without uracil. The colonies of the second screenings were patched to three types of *Pseudomonas* minimal plates with succinate and kanamycin, the master plate with uracil and arginine and selection plates, one with arginine and the other with uracil. Refer to the flow diagram in Figure 5.

The mating and the second screening procedures, developed as outlined above, yielded five *P. putida* auxotrophs unable to grow on *Pseudomonas* minimal medium unless both arginine and uracil were provided.
Since the auxotrophs were resistant to kanamycin, the transposon Tn5 must have inserted into a gene in the pyrimidine pathway creating a mutant strain. This is treated in more detail in the DISCUSSION. Only two of the mutants remained stable during the subsequent testing and the other three were lost. These will now be referred to as the two pyrA mutants.

**Characterization of the pyrA Mutants**

The *P. putida* pyrA mutants were assayed and found to lack carbamoylphosphate synthetase. The mutants, designated G1 and G2, were subcultured each month and maintained on *Pseudomonas* minimal medium with succinate, uracil, arginine, and kanamycin. Since the mutants are almost identical in the testing most of the results discussed do not discern between them. Periodically they were transferred to unsupplemented medium to check against reversion to the wild type. The G1 mutant never showed any reversion, but the G2 mutant occasionally had a few wild colonies which also showed up during control plating in the experiments.

The pyrA mutants were streaked on King's Medium B and incubated for 24 hours at which time they both checked positive for the characteristic greenish fluorescence for *P. putida*. under UV radiation (Palleroni, 1975). Growth on the analog of uracil, 5-fluorouracil, was negative.

To learn if the mutants could grow on media supplemented with other compounds in the pyrimidine pathway, four plates each of *Pseudomonas* minimal medium supplemented with succinate and kanamycin were spread with uracil (control), uridine, cytosine, cytidine, or orotate. Each mutant
FIGURE 5. Flow Chart for Mating and Screening Procedures
1. *P. putida*  
   Overnight
   LB  30°C

2. **Wash** in 100ul  
   0.85% saline

3. **Mating** 100ul  
   YT plates

4. **Incubation**  
   30°C 4 h

5. **Transfer mating mixture**  
   to tubes in 3 ml 0.85% saline

6. **Spread 100ul on screening plates**  
   *Pseudomonas* min medium with  
   suc, arg, ura and Km

7. **Incubation**  
   30°C 48 h

8. **Replica plating on Pseudomonas**  
   min medium with suc and  
   a) master: ura, arg, Km  
   b) arg  
   c) ura
was streaked on two of each type of plate and incubated for 48 hours at 30°C. Since almost no growth occurred, this procedure was repeated with an arginine supplement. Both mutants could grow on minimal plates plus arginine supplemented with uracil, cytosine, cytidine, or uridine but not orotate. As expected, the most growth was on uracil and arginine and somewhat less on cytosine and arginine. Very little growth was found on cytidine and arginine or uridine and arginine. The same results occurred when citrulline was substituted for arginine.

The graph in Figure 6 shows a comparison of the growth properties of the pyrA mutants grown in uracil and arginine or cytosine and arginine. The procedure was carried out 2 different times for each mutant and since very little difference occurred between the two mutants, the four sets of data were averaged together. The standard procedure for obtaining growth curves is described in MATERIALS AND METHODS and the significance of the data collected is in the DISCUSSION. The mutants were also grown in uridine and arginine or cytidine and arginine but growth in liquid medium under these conditions was negative.

Mutants without the Transposon

In an attempt to create true mutants that no longer had the transposon, the following method was developed. The pyrA mutants were grown overnight for five successive days without kanamycin in either LB broth or Pseudomonas minimal medium with succinate, uracil, and arginine to move the transposon out of the pyrA region leaving a mutation there. Screening was done on Pseudomonas minimal plates with succinate that were supplemented with
FIGURE 6. Growth Properties of Uracil or Cytosine (in the presence of arginine). Growth flasks for the Klett spectrophotometer were inoculated with 0.5 ml of the mutant in 50 ml of growth media. Each flask had *Pseudomonas* minimal broth plus succinate, arginine, kanamycin and 40 ug of either uracil or cytosine per ml.
Colony Density in Klett units

Time (minutes)

- Uracil
- Cytosine
uracil and arginine. The colonies were patched to three types of *Pseudomonas* minimal plates with succinate, a master plate with arginine and uracil and two selection plates, one of minimal medium and the other with arginine, uracil and kanamycin. Growth on the master plate but not on the selection plates would indicate the cells were still mutants since they could not grow on media unsupplemented by uracil and arginine but they no longer had the transposon's kanamycin marker. There was always growth on the two types of supplemented plates and a few plates had some growth on the minimal medium as well. After running this procedure seven times with no success, one of my high school students took it over as his research project but also was unsuccessful. The reasons why this might be are given in the DISCUSSION.

II. PROCEDURES for Hfr GENERATION

The mating procedure described earlier was used to reinfect the two transposon mutants with R91-5::Tn5. Each *P. putida pyrA* mutant was mated with *P. aeruginosa* PAO11 pMO75 and PAO11 pMO1896 (reverse orientation). The mating cultures were transferred to screening plates of *Pseudomonas* minimal medium with succinate, uracil, arginine, kanamycin and 1 mg ampicillin per ml. The donor and recipient strains were also transferred to screening plates to check for negative growth. The screening plates were incubated for 48 hours at 30°C.

Screening and selection originally was to be done on carbenicillin/kanamycin plates because the plasmid carries carbenicillin resistance and the
transposon, kanamycin resistance. Since the plasmid is a suicide vector, unable to replicate in the \textit{P. putida} mutants, carbenicillin resistance in the recipients would indicate integration into the chromosome. Refer to Figure 8 in the DISCUSSION. However, \textit{P. putida} PRS2000 had an unforeseen natural carbenicillin resistance. Several antibiotics were tested on the \textit{P. putida} mutants to discover it required 1 mg of ampicillin per ml for counterselection.

Many of the Hfr colonies that resulted for each mutant (G1 and G2) with each donor plasmid (pMO 75 and pMO 1896) were then streaked on \textit{Pseudomonas} minimal plates with succinate, arginine, and uracil. The antibiotics added were 200 ug kanamycin per ml and 1 mg ampicillin per ml. These Hfrs were the ones mated to the \textit{P. aeruginosa} PAO strains with known genetic markers in the next section. The transposon Tn5 in the donor Hfr should mobilize the chromosome at the point of its insertion and if a recombination event occurs in the recipient, transferred chromosomal genes could provide it with a missing marker. Please see the DISCUSSION for further explanation.

III. PROCEDURE to MATE Hfrs to PAO AUXOTROPHS

The \textit{P. aeruginosa} PAO strains were tested for still being auxotrophic for the genetic markers listed in Table 1 of MATERIALS AND METHODS. This was done by streaking the PAO strains on plates of \textit{Pseudomonas} minimal medium with succinate and the supplements listed in Table 2. All six auxotrophs were able to grow if all of the supplements for the genetic markers
were present. However, as shown in Table 2, growth occurred in the PAO18 and PAO944 strains that indicated they no longer had the purine marker. These were not used in the Hfr matings.

Plates of *Pseudomonas* minimal medium with succinate and kanamycin were prepared as described in MATERIALS AND METHODS with requirements added as given in Table 2. Added kanamycin selected for the presence of the transposon. The mating procedures described earlier were used to mate the *P. putida* Hfr donors with the *P. aeruginosa* PAO auxotrophic recipients. Samples of 100 ul of the *P. aeruginosa* recipients were spread on the plates listed in Table 4. The Hfr was designated by which *P. putida* mutant (G1 or G2) it was and also by the *P. aeruginosa* PAO11 donor (pMO75 or pMO1896) that generated it. The four Hfrs of G1(1896) and the four Hfrs of G2 (1896) tested by these procedures gave such inconsistent results that the data are not recorded here. Additional matings with the plasmid pMO1896 Hfrs will be pursued.

Since each procedure required so many matings and 21 different types of plates for each Hfr tested, G1(75) and G2 (75) Hfr matings were run separately from G1(1896) and G2(1896). Each time a procedure was run, two different Hfrs for each of the above designations were mated with each PAO auxotroph, and to validate the results, these procedures were repeated. A total of 20 different Hfrs, 6 for each G1(75), and 6 for each G2(75) and 4 for each G1 (1896) and 4 for each G2(1896) were finally tested. Only the growth of the resultant recipients from the G1(75) and G2(75) donors are recorded in Table 4.

These crosses required a number of controls. *Pseudomonas* minimal medium plates spread with all the growth requirements but without kanamycin were used as controls to ensure that the *P. aeruginosa* PAO auxotrophs were
viable. No growth on a fully supplemented kanamycin plate but growth on one lacking kanamycin meant the transposon had not been transferred but the PAO auxotrophs were still viable. No growth on plates prepared as above but with ampicillin verified that any growth on the test plates could not be the donor Hfrs, the only ones able to grow in ampicillin. The counterselection against the Hfr donor was verified by observing no growth on control plates lacking uracil and arginine. Since the recipients were *P. aeruginosa* they were incubated at $37^\circ$C, which retards any *P. putida* Hfr growth.

Growth on each plate was recorded in Table 4 and the significance of the growth is explained in the DISCUSSION.
TABLE 4. Growth of Recipient Auxotrophs
(min med with supplements listed)

* DONOR Hfrs:  G1(75)  G2(75)
(Number of plates with growth/total 12 Hfrs)

<table>
<thead>
<tr>
<th>PAO RECIPIENTS:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO12:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) ade, leu</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>2) ade</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3) leu</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PAO 949:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) ade, thr, cys</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>2) ade, thr</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3) ade, cys</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4) thr, cys</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>PAO 222:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) met, trp, lys, his, pro, ilv</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>2) trp, lys, his, pro, ilv(-met)</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>3) met, lys, his, pro, ilv(-trp)</td>
<td>0</td>
<td>+1</td>
</tr>
<tr>
<td>4) met, trp, his, pro, ilv(-lys)</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>5) met, trp, lys, pro, ilv(-his)</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>6) met, trp, lys, his, ilv(-pro)</td>
<td>+4</td>
<td>+6</td>
</tr>
<tr>
<td>7) met, trp, lys, his, pro(-ilv)</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>PAO 392:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1) met, trp, lys, pro, ilv, his</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>2) trp, lys, pro, ilv, his(-met)</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>3) met, lys, pro, ilv, his(-trp)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4) met, trp, pro, ilv, his(-lys)</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>5) met, trp, lys, ilv, his(-pro)</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>6) met, trp, lys, pro, his(-ilv)</td>
<td>+6</td>
<td>+6</td>
</tr>
<tr>
<td>7) met, trp, lys, pro, ilv(-his)</td>
<td>+6</td>
<td>+6</td>
</tr>
</tbody>
</table>

* Hfr designations: G1 and G2 are the *P. putida* mutant recipients and (75) designates the *P. aeruginosa* PAO11 pMO75 donor.
CHAPTER 4

DISCUSSION

I. Discovery of pyrA P. putida Mutants

During the mating and screening procedures described in this research, the R91-5 plasmid (pMO75) of the donor, Pseudomonas aeruginosa PA011, was transferred during conjugation to the recipient, P.putida PRS2000. The plasmid pMO75 carries the transposon Tn5, and since the R plasmid cannot replicate in P. putida, Tn5 transposed to the recipient chromosome, inserted into the pyrA gene, and blocked the pyrimidine pathway in both mutants recovered. Insertion of the transposon into a chromosomal site is random and without homology resulting in an array of auxotrophs. Direct selection for mutants capable of growth only on uracil and arginine, with counterselection against the donor and recipient, isolated two mutants unable to synthesize carbamoylphosphate synthetase in the pyrimidine pathway. Refer to the schematic diagram in Figure 7.

Mating and Screening

The original problem of isolating a mutant in Pseudomonas with a defective pyrimidine pathway by transposon mutagenesis seemed simple enough. Not so. After an entire year of developing mating and screening
procedures and observing many thousands of colonies, only two stable mutants were obtained in *Pseudomonas putida*. These were both found to be *pyrA* mutants which are unknown and which there is not any literature that discusses the relative position of a *pyrA* gene on the *P. putida* or *P. aeruginosa* genetic maps.

Many different mating procedures were tried in the beginning of this research with little success, until the experimental procedures done by Bruce Holloway were adapted to this special problem. The final mating and screening procedures that were developed are described in the RESULTS section and will be discussed a little later. In the first two mating procedures run, direct screenings were done for uracil only but since no mutants were obtained, all subsequent selection included both uracil and arginine. As many as 2000 colonies were screened each time a mating procedure was run. Due to the long hours that were required for toothpick patching to selection plates, only one of these mating procedures could be run about every three to four weeks. Several of my high school students volunteered to help patch in the beginning but became disenchanted when no mutants were isolated. During the 4th mating (over 8000 colonies had been screened by now), the first mutant was obtained but it was unstable and eventually lost. During the last four matings, four more mutants were discovered. Two of these proved to be stable *pyrA* mutants and mating procedures were discontinued. Approximately 16,000 colonies had been observed. No wonder these two mutants are rare!

Development of the mating and screening procedures faced several problems. The first matings were done on YT plates with kanamycin (the transposon marker), reasoning that this would yield recipients in which the
Figure 7. Transposition of Tn5. 1. Conjugation between *P. aeruginosa* donor and *P. putida* recipient. 2. The plasmid pMO75 is transferred to *P. putida* using a rolling circle mechanism. 3. The plasmid pMO75 cannot replicate in *P. putida* but Tn5 transposes to the chromosome.
1. **P. aeruginosa**
   - pMO75
   - Tn5
   
   **P. putida**

2. **P. putida**
   - pMO75
   - Tn5

3. **P. putida mutant**
   - Tn5
   - plasmid loss

Transposition of Tn5 from pMO75 results in a Tn5 insertion in the plasmid.
transposon was inserted. However, it became obvious that the recipient cells died before the kanamycin gene could be transferred and subsequent matings were done on YT plates without kanamycin. The selection for kanamycin was done during the screening procedures. To find the minimum concentration of kanamycin that could be used for counterselection of the recipient, a gradient of concentrations, 10 to 1000 ug of kanamycin per ml were added to 10 LB tubes inoculated with P. putida PRS2000 and grown overnight. The 200 ug kanamycin per ml concentration chosen was the same as that used to maintain the transposon in P. aeruginosa PAO11 and later, the two mutants. The inability of the Trp- donor to grow on Pseudomonas minimal medium was also confirmed.

**Tn5 Transposition and Mutagenesis**

The R91-5 plasmid, transferred during conjugation from P. aeruginosa to P. putida, cannot replicate in P. putida increasing the chances that Tn5 will transpose to the P. putida chromosome and mutagenize the cells (Holloway, 1986). Tn5 is a Class I composite transposon which usually uses the conservative method of transposition. Transposase, the enzyme synthesized by Tn5, makes precise staggered cuts at either end of the inverted repeat sequences of Tn5 leaving a lethal gap in the plasmid. The enzyme also makes staggered cuts at a random point in the chromosome, replicating a set of 9 base pairs of the target. It ligates the transposon between these sets of identical target base pairs. Refer to Figure 1 in the INTRODUCTION. This process may insert several copies of the transposon into the chromosome disrupting the function of any gene into which it inserts, causing a mutation (Kleckner, 1981).
Tn5 inserted into the pyrA gene of P. putida creating mutants that cannot synthesize carbamoylphosphate synthetase. This enzyme is necessary in the formation of carbamoylphosphate (H$_2$N-CO-OPO$_3^{-2}$) in the first step of de novo biosynthesis of the pyrimidine ring. However, it is not the first committed step because carbamoylphosphate is necessary in converting ornithine to citrulline in the arginine pathway. Refer to Figures 3 and 4 in the INTRODUCTION. Thus, the mutants can grow only on a minimal medium that is supplemented with both arginine and uracil. A phosphoribosyltransferase reaction that adds uracil to 5-phospho-ribosyl-pyrophosphate yields uridine 5'-monophosphate and PPI in the salvage pathways. UMP can then be converted to UDP, UTP and CTP (Neuhard & Nygaard, 1987).

**Characterization of the Mutants**

The characteristic green fluorescence under UV radiation when grown on King's B Medium affirmed the bacteria as mutants of P. putida. (Palleroni, 1975). They were unable to grow on 5-fluorouracil, an inhibitor of DNA synthesis that blocks the thymidylate synthase conversion of dUMP to dTMP during rapid growth. There are mutants resistant to this analog but these were not (O'Donovan & Neuhard, 1970).

Several different supplements were added to the minimal growth medium in place of uracil to characterize the mutants. At first the supplements were added without arginine but almost no growth occurred, confirming that these mutants lack carbamoylphosphate synthetase. With arginine added, the mutants were still unable to utilize orotate in the medium, even though orotate is converted to orotidine 5'-monophosphate and then to uridine 5'-
monophosphate in the pyrimidine pathway. Refer to Figure 3 in the INTRODUCTION. Whether this means the mutants could not take up orotate or they lack pyrE or another gene in the subsequent pyrimidine pathway was not pursued. Uridine and arginine or cytidine and arginine provided a small amount of growth on all of the test plates, however, growth was so slow that growth curves could not be established for these nucleosides. The predominant route to utilize cytidine in *E. coli* is deamination to uridine by cytidine deaminase in the degradation pathways. Uridine is converted to uracil which is then converted to UMP by enzymes of the salvage pathways in *E. coli* (Neuhard & Nygaard, 1987). Citrulline substituted for arginine gave similar growth characteristics. Since the mutants cannot make carbamoylphosphate, providing citrulline in the arginine pathway allows the formation of argininosuccinate and finally arginine. Refer to Figure 4 in the INTRODUCTION.

With the exception of uracil and arginine, cytosine and arginine added to the minimal medium provided the best growth of the mutants. Transport systems highly specific for both uracil and cytosine have been found in *E. coli*, making it possible for these exogenous bases to be utilized (Neuhard & Nygaard, 1987). Growth on cytosine is less than on uracil, however, because it must first be deaminated to uracil by cytosine deaminase. The growth curves for uracil and cytosine given in Figure 6 of the RESULTS show that uracil is much more efficiently utilized than cytosine.
Mutants Without the Transposon

Several unsuccessful attempts were made to create true \textit{pyrA} mutants that no longer had the transposon inserted in the chromosome. The procedure is described in the RESULTS. After growing the mutants without kanamycin, screening was done on \textit{Pseudomonas} minimal plates with succinate supplemented with arginine and uracil but no kanamycin. If growth had occurred here but not on minimal plates or supplemented plates with kanamycin, they would have been mutants without the transposon. The idea, of course, was to discover an incidence in which Tn5 moved out of the chromosome \textit{imprecisely}, leaving behind a \textit{pyrA} gene with some base pairs missing or adding a base pair. However, there always was growth on both types of supplemented plates indicating that the transposon probably did not move in these cultures. As a Class I transposon, Tn5 most often uses a conservative method which usually leaves a lethal gap behind in the chromosome. If it did use the replicative method it could have left a copy of itself intact and this would be undetectable. Also, if Tn5 moved elsewhere in the chromosome the mutant could have remained an auxotroph that was not being fed the proper nutrients. The few plates that had growth on the minimal medium could be explained as the transposon moving out with precision, leaving behind a normally functioning \textit{pyrA} gene. But since there was growth on the kanamycin plate also, it had to have occurred on the minimal plate only. The above discussion indicates this experiment was doomed to fail from the start but everything is worth a try and it does confirm the stability of the transposon in these mutants and the mechanism of transposition.
II. Generation of Hfrs

Matings between *P. aeruginosa* PAO11 and the mutants reintroduced the plasmid pMO75 into the two mutants resulting in the integration of the plasmid at the site of the Tn5 insertion. The mating procedures are described in the RESULTS. The plasmid was unable to replicate in *P. putida*, hence, it underwent recombination with the homologous region of Tn5 already present in the chromosome (Strom *et al.*, 1990). Since the original insertion of Tn5 was in the *pyrA* gene and very stable, the plasmid is presumed to have inserted here as well. The mutants had now become high frequency of recombination (Hfr) donor strains with the ability to mobilize the chromosome during conjugation. Refer to Figure 8.

During the screening for Hfrs, carbenicillin resistance in the Hfr would have indicated transfer of the R91-5 plasmid but *P. putida* PRS2000 has a natural resistance to carbenicillin so this antibiotic could not be used for this or counterselection. However, the *P. putida* recipient was sensitive to 1 mg ampicillin per ml which could be used for counterselection. Thus, a colony growing on the screening plate of *Pseudomonas* minimal agar supplemented with succinate, arginine, uracil, kanamycin and ampicillin was an Hfr since the recipient mutant could not grow on the ampicillin. The reasoning here is that the Hfr could grow on the ampicillin since it had the natural carbenicillin resistance enhanced by the resistance carried by the R91-5 plasmid.
These Hfrs were generated in order to locate the position of the pyrA gene on the Pseudomonas chromosome map. The transfer origin of the R91-5 plasmid is the pyrA gene and any genes located close by could be transferred during conjugation. Refer to Figure 8.

III. Mating pyrA Hfrs to P. aeruginosa Auxotrophs

The Hfr donor strains of the Tn5 pyrA mutants of P. putida should allow the mapping of pyrA on the P. putida genetic map. When the Hfr donor is mated with an auxotrophic recipient with a known genetic marker, the Hfr mobilizes the chromosome, transferring the genes closest to the origin of transfer. A recombination event in the auxotrophic recipient is detected as colonies on a plate lacking the supplement for that marker. Since the origin of transfer is the Tn5 insertion in pyrA this would give the approximate location of the pyrA gene (Strom et al., 1990). Unfortunately, P. putida recipients with known genetic markers were not available at the time of this study and P. aeruginosa PAO strains were used instead. The consequences of this will be discussed.

Since there was so much growth on the first screening plates, there was concern in the beginning of these experiments that the colonies appearing were not those of the PAO recipients of the pMO75 plasmid, but colonies of the parents. The P. aeruginosa PAO strains were tested for still being auxotrophic for the genetic markers listed in Table 1 and PAO18 and PAO944 were not used in subsequent matings. The mating procedures described in the
RESULTS were controlled as follows. The plates with all the supplements were control plates. Refer to Table 4. The control plates without kanamycin ensured that the recipients were able to grow, but with kanamycin, showed that the transposon was transferred even if growth on the screening plates was negative. Although counterselection against the Hfr was the minimal medium without uracil, a further precaution against the Hfr was to transfer it to minimal medium with kanamycin and ampicillin. Since only the Hfr was Amp\textsuperscript{R} any growth would mean the Hfr could grow on these plates, but growth was negative. Still other controls transferred the donor and recipient to the screening plates directly. The final conclusion drawn from all these controls was that the colony growth recorded in Table 4 was that of PAO recipients that had received Tn5 and at least part of a mobilized chromosome.

The data collected in Table 4 verifies that the Hfrs have been generated from the \textit{pyrA} mutants but there is no conclusive evidence of where the \textit{pyrA} gene might be located on the \textit{P. aeruginosa} genetic map. Except for one plate in PAO222 (that could have been an anomaly) there was no growth on the trp-plates. This cannot be a problem with the trp supplement because there was growth on the control plate. Thus, the gene for Trp was never transferred. Refer to Figure 9. It would be nice to say that \textit{pyrA} is located somewhere in this region at 35 min and Tn5 is mobilizing the chromosome in the direction of \textit{met-28}. But then, that does not explain why there was never growth (except one plate) for the \textit{pur-136} at 25 min. The data is just too inconclusive and there are several reasons for this. The mating time had to be increased longer than probably is advisable for these types of experiments. The PAO strain grows at 37\textdegree C and the Hfr strain grows at 30\textdegree C, thus the mating took place at the lower
temperature but with increased time. This was successful in the original matings for transposon mutagenesis but perhaps not here. Tn5 can orient in either direction with an 8:1 preference for one direction (Strom et al., 1990) but no indication of that was shown here. I was unable to find any experiments where the recipients were not of the same species as the Hfr donors. Perhaps, using \textit{P. putida} strains would have been more efficient. Theoretically, the mapping experiment should work, but practically it did not.

This work will continue with \textit{P. putida} recipient strains with known genetic markers and interrupted mating procedures. The lack of \textit{pyrA} mutants and a \textit{pyrA} marker on any \textit{Pseudomonas} genetic map raise the hope that the defective \textit{pyrA} genes found in these mutants will eventually be placed on the \textit{P. putida} genetic map.
Figure 8. Conjugation and Recombination. 1. During conjugation, the plasmid pMO75 transfers to the *P. putida* mutant. 2. The plasmid is unable to replicate in *P. putida* but undergoes recombination with the homologous region of Tn5 already present in the chromosome. 3. The entire plasmid inserts in the region of the *pyrA* gene and the *P. putida* mutant becomes a high frequency of recombination (Hfr) donor. 4. During conjugation between the Hfr and an auxotrophic recipient with a known genetic marker, the Hfr mobilizes the chromosome, transferring the genes closest to the origin of transfer, the Tn5 insertion in *pyrA*. 
1. Conjugation of \textit{P. aeruginosa} with \textit{pMO75} and \textit{Tn5} into \textit{P. putida} mutant 2. Recombination

4. Recipient recombinant for defective gene C
Figure 9. *Pseudomonas aeruginosa* Chromosome Map
(from Ratnaningsih et al., 1990).
The two lines on the right have the locations of the genetic markers of the auxotrophs used in this study.


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