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PROJECT REPORT

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Cloning *ArgF* and *ArgG* genes of *Pseudomonas aeruginosa*

By

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SUMMARY

The R prime plasmid, pCR1 carrying *argF/G* genes was isolated in large scale from *Pseudomonas putida* PPN1092 strain by a modified Wheatcroft and Williams (1981) method. The fractions obtained from the sucrose gradient contained reasonably pure but rather dilute solutions of the plasmid DNA. Attempts to concentrate plasmid DNA by acetate-ethanol precipitation resulted in loss of the plasmid.

The pCR1 plasmid was digested with restriction enzymes, PstI and BamHI and the fragments were ligated with similarly restricted and dephosphorylated plasmid vectors, pAT153 and pBR322.

Attempts to transform *E.coli* W4100 Arg$^-$strain by the recombinant plasmid vectors as well as the whole plasmids, pAT153 and pBR322 were not successful. Hence, an easily transformable strain, *E.coli* HB101 was taken and transformed. Recombinant plasmid pBR322 failed to transform the competent cells whereas pAT153 carrying pCR1 DNA fragments both at PstI and Bam HI sites (separately) transformed the cells with fairly high transformation frequencies (7.56×10^{-6} and 3.24×10^{-6}, respectively).

210 transformants from cells treated with pAT153 carrying inserts at the PstI site and 252 from those trans-formed with the same plasmid with inserts at Bam HI site were isolated out of which 73 (pPK1 - pPK73) and 78 (pBK_1 - pBK78) colonies, respectively were found to be recombinants (i.e. about 35% and 31% recombination).

Plasmids were isolated from these recombinant strains (pPK and pBK strains) by Holmes and Quigley's (1981) method and pooled in different batches. These pooled recombinant plasmid DNA preparations were used for transforming *E.coli* W4100 Arg$^-$strains. The preparations from pPK strains (i.e. pAT153 carrying pCR1 fragments at Pst I site) failed to transform the competent cells whereas all the batches of plasmid pools from pBK strains (i.e. pAT153 with pCR1 fragment inserts at Bam HI site) transformed the cells with varying transformation

frequencies (2.98×10^{-6}, 1.25×10^{-6}, 2.11×10^{-6} and 2.39×10^{-6}).

57 representative recombinant colonies (pBMK strains) with phenotypes Arg^{+}, Amp^{R} and Tet^{s} were isolated and subcultured on selective media and the phenotypes were confirmed. These colonies, however, were slow growing.

Efforts to isolate recombinant plasmid DNA from these strains were not successful.

CHAPTER 1. - INTRODUCTION

Microbial processes for the production of a number of metabolites have been known and been practised for centuries and in the last few decades they have made significant contribution to industrial development. However, there has been an ever increasing awareness about the immense potentialities of biotechnological processes in recent years. Improvement of the yield of various microbial products such as antibiotics, enzymes, organic solvents, amino acids, and a wide variety of other valuable products has been achieved by various means. They include, mainly, the selection of potent microbial strains and optimization of various nutritional and cultural conditions so as to enable overproduction of the desired product. Another approach has been the genetic manipulation of the producing organism for improving the yield of the desired product (Malik, 1979).

Genetic Manipulation: This primarily involves strain improvement through mutagenesis and selection of mutants resistant to feedback inhibition or repression. Various physical agents such as ionising radiations and chemicals are used for bringing about mutations. Many of the microbial strains used at present in industry for the production of a number of microbial metabolites were developed by this method.

Protoplast fusion is another technique which is proving successful in strain improvement. Somatic hybridization that results after fusion of cells having different genetic configuration enables them to increase the genetic recombination of genes coding for different desired characters. For example, a high producing but slow growing mutant could be recombined with a fast growing strain - both coming from the same ancestor prototroph - which would result in the formation of high yielding and fast growing strain. The technique has been very successfully made use of in the so called 'hybridoma technology' particularly for the production of monoclonal antibodies. This technique has also been tried in the case of fungal and streptomycete strain improvement programmes.

Recombinant DNA technology is yet another genetic manipulation technique which is becoming increasingly significant. Gene amplification is one of these techniques which involves cloning of a full or part of the structural gene into a multicopy cloning vector (eg. a plasmid) which, in turn, is introduced into the cell. This helps to enhance the production of the gene product by the increased rate of expression of the gene due to better defined means of gene regulation. In other words, recombinant DNA technology comprises tailoring the gene by selectively cutting (restricting) and joining (ligating) the different pieces together as desired and eventually inserting the tailored genome into the cell. By this technique it has been possible to introduce foreign genes from genetically very different organisms (including mammalian DNA) to microorganisms. *Escherichia coli*, a Gram-negative bacterium of alimentary canal is the host organism which has been extensively used for cloning of foreign genes. Recently genetic engineers have been looking for other organisms as an alternative for *E.coli* which has some disadvantages such as potential health hazard (being a normal flora of intestine), inability to excrete the gene product, lack of protein modification system which is required for the formation of active eukaryotic gene products etc. *Bacillus subtilis* and *Saccharomyces cerevisiae* have proved to be of use as host organisms for cloning foreign genes. Members of the genus, *Pseudomonas* are another group of organisms which have attracted increasing attention, recently for genetic studies.

Genetics of Pseudomonas The special attraction towards the studies on *Pseudomonads* was mainly due to their biochemical diversity, significance for infectious disease and their resistance to antibiotic therapy, and increasing awareness that their genetic constitution is different from other bacteria. They are capable of utilising a wide variety of substrates as carbon and nitrogen sources (Chakrabarty, 1976). A lot of work has been done on the chromosomal as well as extrachromosomal genetics of *Pseudomonas* in various laboratories. Excellent reviews have been published on the genetics of

Pseudomonas (Holloway *et al*, 1975; Stanisich and Richmond, 1975; Holloway, 1979; Bagdasarian and Timmis, 1982; Sakaguchi, 1982; Clarke, 1984; Clarke and Richmond, 1975).

(i) Chromosomal genetics For over 30 years extensive work has been done on the detailed mapping of *E.coli* chromosomal DNA. However, mapping of *Pseudomonas* chromosome is of rather recent origin. Earlier studies were made on two strains of *P.aeruginosa*, PAO and PAT (Holloway, 1969; Watson and Holloway, 1978; Holloway *et al.*, 1979). In a more recent study a third strain has been subjected to gene mapping (Sambanthamurthi, 1983).

Three processes of Chromosomal transfer: conjugation, transduction, and transformation are found in *Pseudomonas* among which conjugation is the most useful.

(a) Conjugation. A number of plasmids have been used in the mapping of the chromosomes of *P.aeruginosa* strains. FP_2, the sex factor was the first plasmid to be used in the mapping of *P.aeruginosa* strains PAO and PAT (Holloway, 1955). Later a number of similar plasmids having the same site of origin for chromosome transfer (defined as zero minutes) have been isolated which includes FP_5, FP39 (Matsumoto and Tazaki, 1973; Pemberton and Holloway, 1973) and FP110 which transfers chromosome from an origin around 25-28 minutes, in the opposite direction to FP2 (Royle and Holloway, 1980). However, it was not possible to establish the circularity of the chromosome by using these plasmids as they have only one site of origin.

A number of R plasmids belonging to Inc P-1 group and having wide host range (Datta and Hedges, 1972) were shown to be having good Chromosome mobilising activity (Cma) in *P.aeruginosa* strain PAT (Watson and Holloway, 1978). Inc P-1 plasmids were used in conjunction with R91, and Inc P-10 plasmid to demonstrate the circularity of the PAT chromosome. They had poor Cma in *P.aeruginosa* PAO. However, isolation of variants of R68 (Inc P-1 group) with higher Cma such as 68.45 (Haas and Holloway, 1976; and 1978) led to the detailed mapping of strains

of *P.aeruginosa* and particularly to the establishment of circularity of PAO chromosome (Royle *et al*, 1981). Fig.1 shows the chromosome map of *P.aeruginosa* strain PAO (Holloway and Crockett, 1982).

Earlier works on the mapping of *P.putida* chromosome were not quite successful as plasmids used such as K (part of OTC plasmid), CAM the Camphor utilising plasmid, pfdm etc., showed very low Cma. Later Martinez and Clarke (1975) showed that R68.44 could promote chromosomal transfer in *P.putida* which was followed by the isolations of a number of enhanced chromosomal transfer (ECM) plasmids possessing high Cma (Nayadu and Holloway, 1980). In 1983 Dean and Morgan were able to establish the circularity of *P.putida* chromosome with the help of Hfr donors formed by the integration of R91-5:: Tn501 into the chromosome. Fig. 2 shows the chromosome map of *P.putida* PPN.

Chromosome transfer has been shown to be promoted by ECM plasmids, especially by R68.45, in other bacterial genera too e.g. *E.coli*, *Klebsiella pneumoniae*, *Rhizobium phaseolii*, *Methylophilus methylotrophus* etc. (Haas 1983)

(b) Transduction. Propagaton of bacteriophages in *P.aeruginosa* is common. A number of such phages have been characterised which include B_3 and F116 (Holloway *et al.*, 1960), G101 and a variant of F116 called F116L (Krishnapillai, 1971). They are capable of determining the chromosomal order of closely linked markers. *P.aeruginosa* has been found to be lysogenic for at least one phage. Phages F116L and G101 which are generalised transducing phages have been extensively used to mapping of the *P.aeruginosa* chromosome. Transducing phages for other species of *Pseudomonas* are much less common. However, phages such as Ø12 and ØW-14 in *P.acidovorans*, M3 in *P.maltophilia*, pf16 in *P.putida* have been isolated (Holloway *et al*, 1979).

(c) Transformation. Transformation of chromosomal markers, although with low frequency, has been reported for *P.putida* (Mylroie *et al*, 1978) and *P.solanacearum* (Boucher and Sequeira, 1978). However, it has not been possible for *P.aeruginosa* though plasmid transformation of Ca^{2+}-treated recipients has

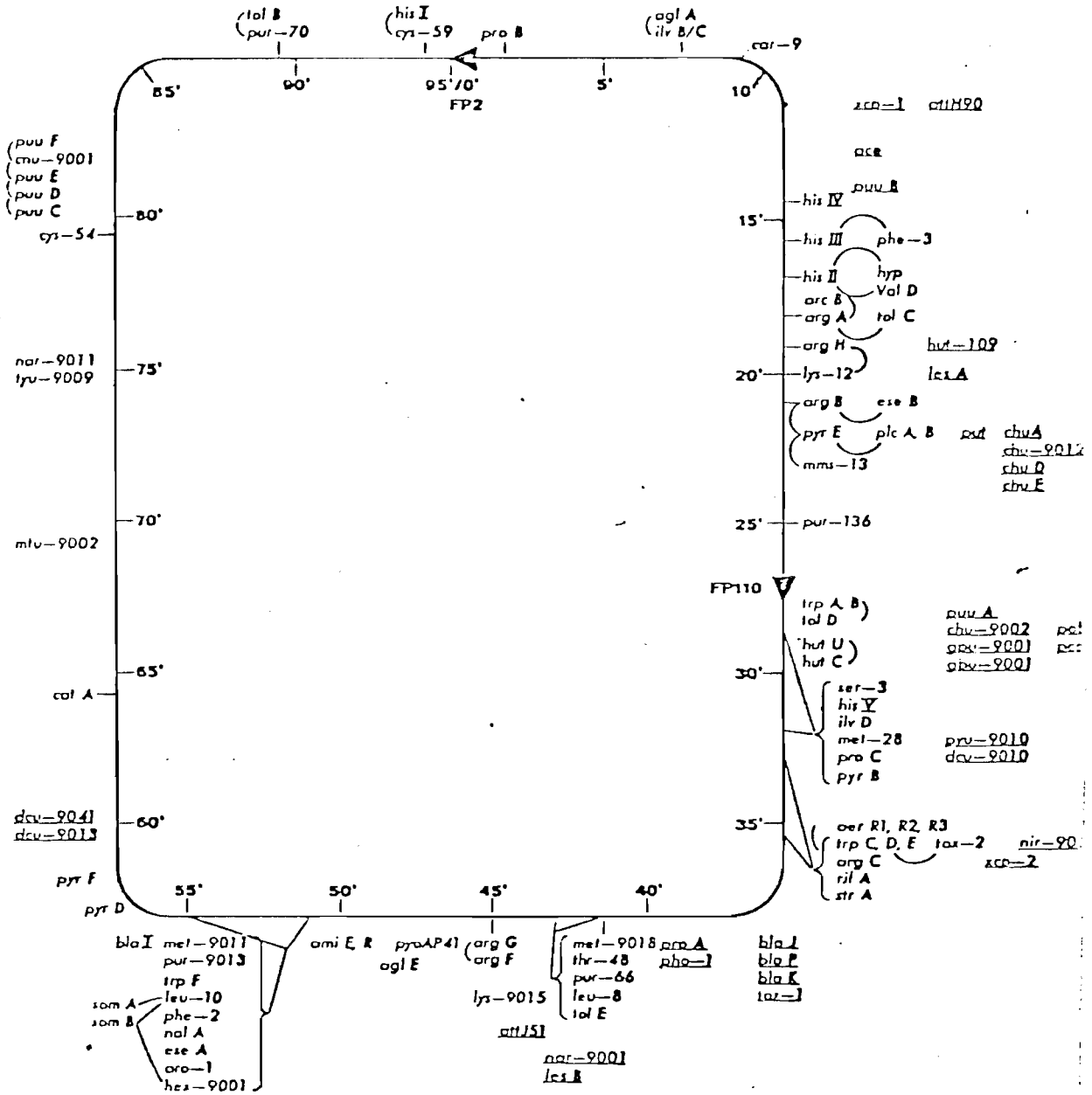


Figure 1. Chromosome Map of *Pseudomonas aeruginosa* PAO (taken from Holloway and Crockett, 1982)

been reported (Sano and Kagegama, 1977; Sinclair and Morgan, 1978). It may be because the linear chromosomal DNA is degraded in *P.aeruginosa* as in the case of *E.coli* (Holloway *et al*, 1979).

(ii) Plasmids in Pseudomonas: Plasmids ranging from tiny cryptic entities through resistant plasmids and sex factors to large degradative plasmids have been reported in *Pseudomonas* (Chakrabarty, 1976); Jacoby and Shapiro, 1977; Haas, 1983).

(a) FP-plasmids The first plasmid to be discovered in *P.aeruginosa* was the sex factor FP-2 (Holloway and Jennings, 1958). Besides the ability to promote chromosome transfer in conjugation this and other sex factors such as FP-5 in *P.aeruginosa* confer resistance to mercuric salts (Loutit, 1970 ; Matsumoto and Tazaki, 1973). Another sex factor, FP39 is different from others in that it cannot confer mercury resistance though it is capable of conferring a Leu^+ phenotype in Leu^- strains of *P.aeruginosa* (Holloway, 1975). Sex factor FP110 does not have any apparent phenotype other than Cma in *P.aeruginosa* (Royle and Holloway, 1980).

(b) R-Plasmids R plasmids which were originally detected independently by Lowbury *et al.* (1969) and Black and Girwood (1969) were extensively studied later after the observation that they were responsible for Carbenicillin resistance (Sykes and Richman, 1970; Fullbrook *et al*, 1970).

Attempts have been made by many workers to determine the physical characteristics of the plasmids. R plasmids RP1 and RP8 are similar in their buoyant densities (1.719 g/Cm^3) and have a 60% value for G+C (guanine + cytosine). 100% homology to immobilised RP8 was shown by RP4, RP1 and RP8. In a reciprocal experiment, however, RP8 showed only 65% homology with RP1. This was because RP8 was 22 megadaltons (Md) larger than RP1 which was 40Md (Grimsted *et al.*, 1972; Saunders and Grimsted, 1972). RP1, RP4, R68 and RK2 were reported to be identical and had a molecular size corresponding to 56.4 Kb (40 Md) (Burkardt *et al.*, 1979) whereas RP8 was larger (93 Kb or 62 Md. Thomas, 1981).

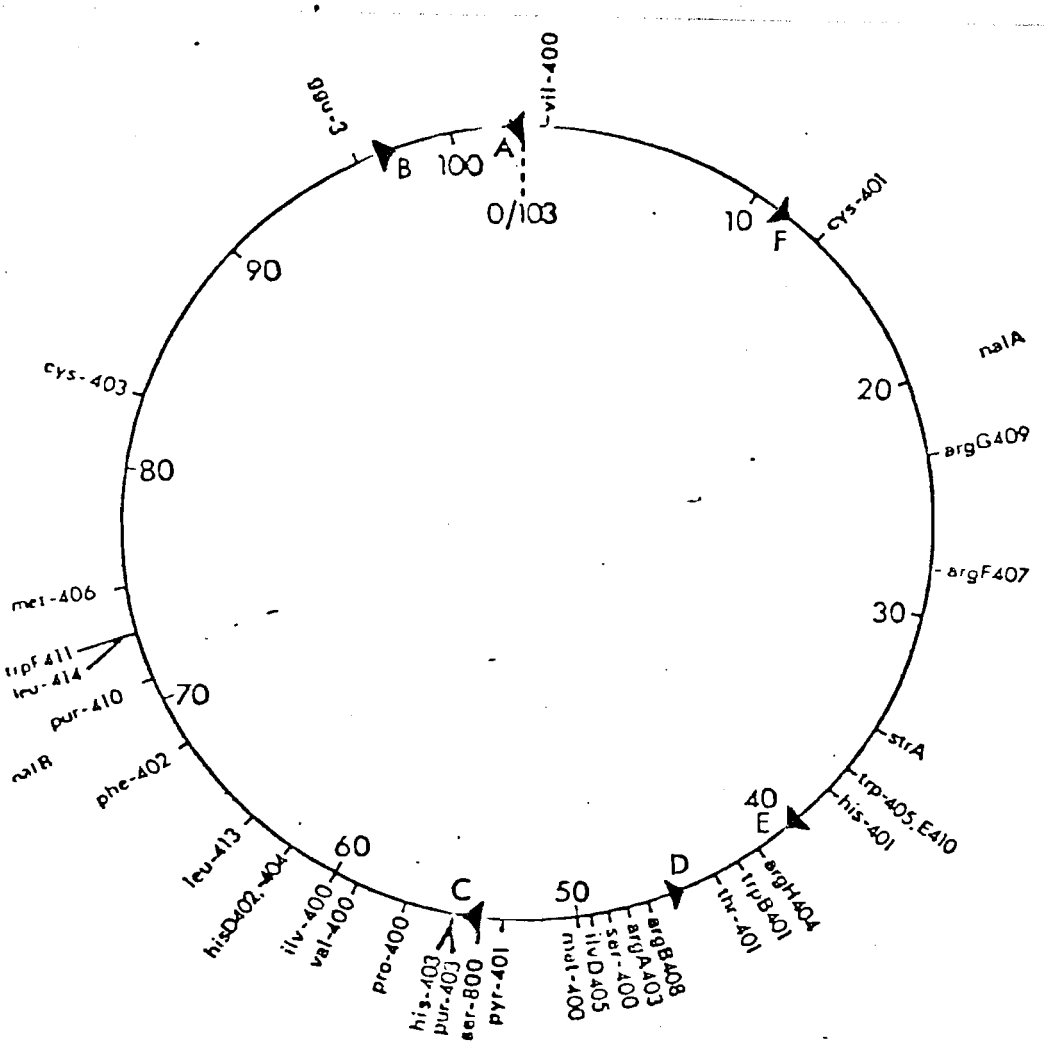


Figure 2. Chromosome Map of *Pseudomonas putida* PPN (taken from Dean and Morgan, 1983).

R68 was used for mapping *P.aeruginosa* PAT chromosome. But it showed low Cma in strain PAO. However, a derivative of this plasmid, R68.45 was shown to have multiple sites of origin in both the strains as well as in various other Gram-negative bacteria (Holloway, 1979). This plasmid was shown to have acquired an additional insertion sequence of 2.12 Kb (Jacob *et al.*, 1980; Riess *et al.*, 1980).

(c) R-Prime Plasmids. R68.45 plasmids carrying fragments of chromosomal DNA have been isolated from a variety of host species such as *P.aeruginosa* (Holloway, 1978) *P.putida* (Morgan, 1982), *E.coli* (Hedges and Jacoby, 1977) and *Methylophilus methylotrophus* (Moore *et al.*, 1983). The Cma of R68.45 is believed to be related to a duplicated section of plasmid DNA sequence in R68 (the 2.12 Kb segment called *IS21* which has the properties of an insertion element) (Riess *et al*, 1980). The chromosome mobilization occurs by means of a cointegrate intermediate formed during transposition of *IS21* into the chromosome (Willetts *et al.*, 1981). In R68.45 prime plasmids carrying *P.aeruginosa* DNA, the chromosome insert is flanked by direct repeats of the 2.12 Kb element (Leemans *et al.*, 1980).

Deletions of the DNA fragments from RI-plasmids were observed after conjugation. This usually happened starting between the two copies of the insertion sequences and extending to the integrated chromosomal fragment (Depicker *et al.*, 1980, Haas and Riess, 1983). In this process loss of markers such as antibiotic resistance was observed. According to Morgan (1982) this loss of antibiotic resistances in newly isolated R-primes was analogous to the instability of plasmids seen after chromosome mobilization. This happens, according to him, after transfer of the R-prime to its recipient host but prior to the establishment of a stable R-prime plasmid.

In a number of other species also R-primes have been formed using R68.45 (Rice, 1984).

Detailed studies for elucidation of physical, physiological and genetic

characteristics of various R-prime plasmids have been carried out recently by Rice (1984). The plasmids pM0780 and pAS13 which were previously derived from pM061 with chromosomal inserts were shown to include the *amiER* genes and the *argFG* genes. By studing the nutritional requirements she was able to obtain *ami*$^-$ drivatives of pM0780 (pM0780I - {*ami*$^-$}) and pAS13 (pCR1 or pAS13 Ami$^-$). Differences in structure were shown to correlate with changes in the genetic composition. The size of all R-primes she investigated were seen to be larger than 100 Kb. By Southern blotting techniques using λ*ami* probe she was able to establish the fine structure map of the amidase gene segment and the surrounding DNA in pAS13 which correlated with results of other workers (Clarke *et al*, 1981; Cousens and Drew, 1984) and new data concerning regions of DNA upstream of the 5' end of the *amiE* structural gene were accumulated.

(d) Degradative Plasmids of Pseudomonas. A number of plasmids which were the first to be isolated from *Pseudomonas* have been named as degradative plasmids (Chakrabarty, 1973). Most of them code for the catabolism (degradation) of aromatic organic compounds (Chakrabarty, 1976; Don and Pemberton, 1981; Yen and Gunsalus, 1982; Cane and Williams, 1982) eg. xylene, toluene(TOL), alkane (OCT), Camphor (CAM), salycylate (SAL), naphthalene (NAH), and insecticides (pJP3). The catabolism of most of these compounds are via catechol or substituted catechols and through meta pathway. Many of these plasmids are self-transmissible and others are not. The size may vary from 50 to 200 Kb.

(e) Hybrid Plasmids Host range of specific plasmids could be extended by constructing hybrid plasmids. Transfer of at least part of an Inc P-2 plasmid,an R plasmid to *E.coli* and other enteric bacteria (Jacoby *et al.*, 1976; Stanisich and Bennett, 1976) was facilitated by hybridizing an Inc P-1 and Inc P-2. Similarly, transposition of TOL genes into RP4 allowed the gene expression in *E.coli* host in which case the expression was low (Jacoby *et al*, 1978). Clarke and Laverack (1983) were able to transfer R$^{\text{w}}$-plasmids carrying *argF* genes from *P.aeruginosa* strains PAO and PAC to *P.putida argF* and *E.coli*

strains. The expression of this gene in *Pseudomonas* was very good whereas it was very low in *E.coli*. Chakrabarty *et al.* (1978) have studied transposition of plasmid DNA segments specifying hydrocarbon degradation and their expression in various microorganisms and found that the TOL from RP4:: TOL could be transposed on to other R plasmids and that RP4::SAL hybrids could be obtained.

In most of the cases studied where hybridization was carried out between Inc P-1 and Inc P-2 plasmids, Inc P-1 properties were found to be "dominant".

It has also been possible to transfer hybrids composed of Inc P-1 plasmid Mu to a number of genera including *Escherichia, Klebsiella, Pseudomonas, Rhizobium, Serratia, Proteus,* and *Erwinia* (Holloway, 1979).

(iii) Cloning Vehicles

(a) Plasmid Vectors There are a number of reviews on plasmid vectors (Bolivar, 1979; Bolivar and Backman, 1980; Kahn *et al.*, 1980). The first plasmid to be used in a cloning experiment was pSC101 (Cohen *et al.*, 1973). The criteria that have to be fulfilled for use of plasmid vectors are that the insertion should not interfere with replication of the plasmid and it should not disrupt the marker gene. Plasmids of minimal size with 2 or 3 markers in order to permit cloning by insertional inactivation are, however, preferred (Thompson, 1982). Plasmids pBR322 and pACYC184 and their derivatives are the most commonly used plasmid cloning vectors. These plasmids have been very useful in *E.coli* and closely related enteric bacteria. However, they are not very well maintained in *Pseudomonas*. Hence, new vectors were developed.

As mentioned in the previous section a number of hybrid plasmids have been found to be useful for this purpose. A number of derivatives of these were also developed (Windass *et al.*, 1980).

Small multicopy Inc Q/P-4 plasmids which can be mobilised by transfer-proficient plasmids such as RP1 (carrying streptomycin and sulphonamide

resistances (Sm, Su) were also developed (Grinter and Barth, 1976). They included RSF1010 and R300B. Derivatives of RSF1010 have also been developed (Bagdasarian *et al.*, 1981).

(b) Phage Vectors: Lambda (λ) has been used to develop a number of vectors though it has the limitation of restriction on the size of the foreign DNA insert. The maximum additional DNA that could be added is only 22 Kb as there are 30 Kb of essential genes. Both replacement and insertion vectors have been developed.

A Lambda, λL47 having a central replaceable fragment bounded by *BamHI* targets, with a further 8 Kb capacity provided by two deletions was reported by Loenen and Brammar (1980). This was employed to clone the amidase genes of *P.aeruginosa*. Karn *et al.*, (1980) and Rimm *et al.*, (1980) have reported the use of λ1059 and Charon series of coliphages respectively based on a similar concept.

An insertion vector, λ641 which can carry small fragments of DNA was constructed by Murray *et al.*, (1979).

A multipurpose cloning site inserted into β-galactosidase gene which has been attached to the single stranded DNA phage, M13 was developed by Messing *et al.*, (1981) which could be used for shotgun DNA sequencing.

(iv) Gene Expression and Regulation in Pseudomonas:

Some detailed studies on gene expression in *Pseudomonas* have been done with special respect to the amidase system (Brammar and Clarke, 1984). Catabolite repression of amidase synthesis could be partially relieved by the additions of an inducer. Stimulative effect of cyclic adenosine monophosphate (cAMP) in the amidase synthesis has been observed by Smythe and Clarke (1975) in both inducible and constitutive strains. The structural amidase gene (*amiE*) is considered to be under positive control (Farin & Clarke, 1978).

The enzyme amidase in *P.aeruginosa* has been used as a model for studies in experimental evolution (Clarke, 1984).

(a) Arginine Metabolism and its Regulation in Pseudomonas.

The biochemical steps of arginine synthesis is schematically shown in Fig.3. Step 5, i.e. from N-acetylornithine to ornithine is carried out in *Pseudomonas* by a transacetylation reaction with glutamate which results in the production of ornithine and N-acetylglutamate (Udaka, 1966). Similar reaction occurs also in other organisms such as *Micrococcus glutamicus*, *Saccharomyces*, *Neurospora* and *Chlamydomonas*. As could be seen in the figure this reaction in which N-acetylglutamate is formed at the expense of the deacetylation of N-acetylornithine, by-passes the enzyme A. Organisms possessing ornithine transacetylase have another feedback inhibition site ie. enzyme B, N-acetylglutamate 5-phosphotransferase, but not in those organisms possessing N-acetylornithine deacylase (Udaka, 1966). Isaac and Holloway (1972) observed that arginine and citrulline (also to some extent ornithine) inhibited enzyme B in cell free extracts of *P.aeruginosa*. Leisinger *et al.*, (1972) confirmed the inhibition by arginine of this enzyme using a highly purified preparation. Haas *et al.*, (1972) showed that enzyme A from *P.aeruginosa* also was being inhibited by arginine. Thus it could be seen that arginine biosynthetic pathway is controlled very strongly by feedback inhibition.

The genes coding for enzymes in the argine biosynthetic pathway are believed to be widely scattered around the chromosome as evidenced by the work of Feary *et al.*, (1969) who found that arginine auxotrophic mutants of *P.aeruginosa* could be separated by transductional analysis into seven separated linkage groups. Under conditions of deprivation or excess of arginine in the medium Isaac and Holloway (1972) did not find significant variation in the levels of enzymes B, E and H (N-acetylglutamate 5-phosphotransferase, N-acetyl-ornithine glutamate transacetylase and arginosuccinase, respectively). It is only *argF*, coding for biosynthetic ornithine carbamoyl-transferase (Ornithine carbamylase or OTCase) that is severely repressed by arginine (Isaac and Holloway, 1972). However, significant derepression was observed during

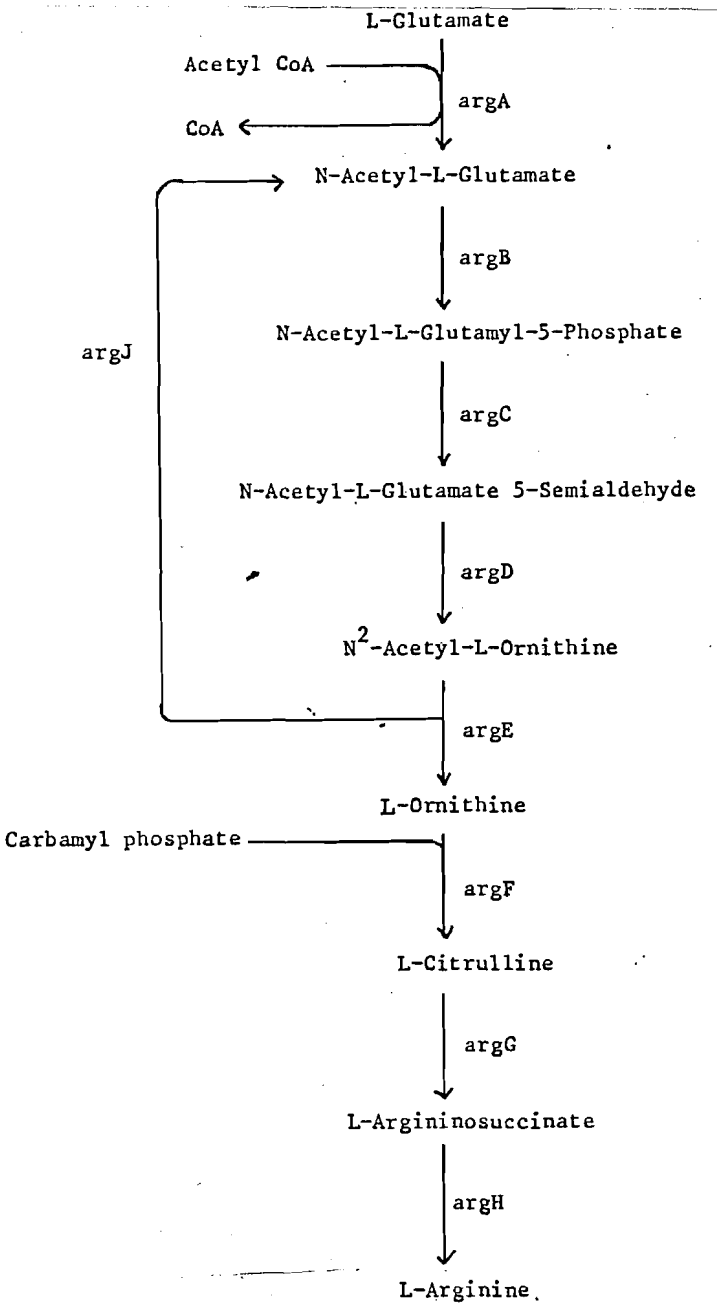


Figure 3. The biosynthetic pathway for arginine in *P.aeruginosa* (Isaac Holloway, 1972; Leisinger *et al.* 1972). The following gene designations are used: *argA* - N-acetylglutamate synthase; *argB* - N-acetylglutamate 5-phosphotransferase; *argC* - N-acetylglutamate 5-semialdehyde dehydroganase; *argD*- N^2-acetylornithine 5-amino transferase; *argE* - acetyl ornithinase; *argF* - anabolic ornithine carbamyltransferase; *argG* - arginino succinate synthase; *argH* - argininosuccinase; *argJ* - ornithine acetyl transferase.

Enzyme A and B are inhibited by arginine.

Enzyme F is repressed by arginine.

exponential growth when grown in minimal medium. The derepressed level of OTCase was not higher in arginine auxtrophs than in the wild type. Voellmy and Leisinger (1972) showed 50-fold repression of OTCase and 2-fold N-acetylglutamate semialdehyde dehydrogenase by arginine. They also observed repression of these two enzymes when a double auxotroph was grown either with limiting leucine or with limiting arginine. However, N-acetyltransaminase was enhanced 15-fold by arginine whereas no significant change was observed in the specific activities of other enzyme of the pathway.

Recently, Haas *et al.*, (1977) have identified six loci coding for arginine biosynthetic enzyme in *P.aeruginosa* PAO by enzyme assay. They were *argA* (N-acetylglutamate synthase) *argB* (N-acetylglutamate 5-phosphotransferase). *argC* (N-acetylglutamate 5-semialdehyde dehydrogenase) *argF* (anabolic ornithine carbamoyltransferase or OTCase) *argG* (arginosuccinate synthetase) and *argH* (arginosuccinase). As reported earlier they also found that these genes were widely scattered. However, they were able to map *argA*, *argB* and *argH* on a short chromosome segment (approx. 3 min long). They also found that *argF* and *argG* were cotransducible indicating their close alignment in the chromosome. As mentioned above *argF* is subject to severe regulation by feedback repression. Though it is believed that the regulation of amino acid biosynthesis in the *Pseudomonads* is different from that of the enteric bacteria such as *E.coli* there is very little detailed information of promoters of *P.aeruginosa*. Certain mutations have resulted in high yields of amino acid and products (Sambantha-murthi *et al.*, 1984).

Professor Clarke's group at University College London have observed, during the course of their studies on the amidase genes of *P.aeruginosa*, 20 to 30% linkage of *amiE* to *argF* and they have obtained R (prime) plasmids carrying amidase genes by selecting for *argF* (arginine prototrophy) and screening for amidase activity (Clarke and Laverack, 1983). They studied the expression of *argF* genes carried by R (prime) in strains of *P.aeruginosa*, *P.putida* and *E.coli* and found that though transfer of these plasmids resulted in full expression

of the gene in both *Pseudomonas* spp. the expression in *E.coli* was only 2 to 4% of that in *P.aeruginosa*. Exogenous arginine did not repress the expression of *argF* gene unlike in the case of *Pseudomonas* strains and also there were no significant differences between $argR^+$ and $argR^-$ *E.coli* strains (*argR* is the regulator gene for arginine biosynthesis in *E.coli*).

Modi (1984) has made some efforts to clone restriction fragments of a R' plasmid, *pCRI* carrying *argF* and *argG* into *E.coli*. A double digest of the purified plasmid DNA (*pCRI*) was prepared by treatment with *EcoRI/SalI* or with *BamH1/Sal1*. These fragments were then ligated into the vector pAT153 which was similarly restricted. The recombinant plasmid was transformed into *E.coli* JA221 with a transformation frequency of 2.9×10^{-6} transformants/µg DNA (vector). He obtained 134 transformants by selection for antibiotic resistance. However, on later screening none of these recombinants were found to carry the *argF/argG* genes (R.E. Drew, personal Communication).

(v) Catabolism of arginine Many *Pseudomonas* species are capable of utilising arginine as the sole source of carbon and nitrogen. The initial steps in breakdown of arginine in *P.aeruginosa* involves 3 enzymes, arginine deaminase, ornithine transcarbamylase (catabolic OTCase) and carbamate kinase (Fig. 4.a & b). Other catabolic pathways also may occur (Mercinier *et al.*, 1980; Rahman *et al.*, 1980; Haas, 1983). Catabolic OTCase of *P.aeruginosa* is different from the enzyme involved in the biosynthesis of arginine. OTCase (catabolic) has neutral pH optimum while the biosynthetic OTCase has a broad optimum around pH 8.5. The OTCase (catabolic) of *P.aeruginosa* is induced by arginine while the biosynthetic OTCase is strongly repressed, as mentioned earlier. But all the three arginine catabolic enzymes in *P.fluorescens* were found to be constitutive (Stalon *et al.*, 1967; 1972).

(vi) The Objectives and the Strategy of the Project.

The above information offers a background to develop a strategy for future studies on arginine biosynthesis which may lead to the construction of strains capable of overproducing the amino acid, arginine. A possible strain for this

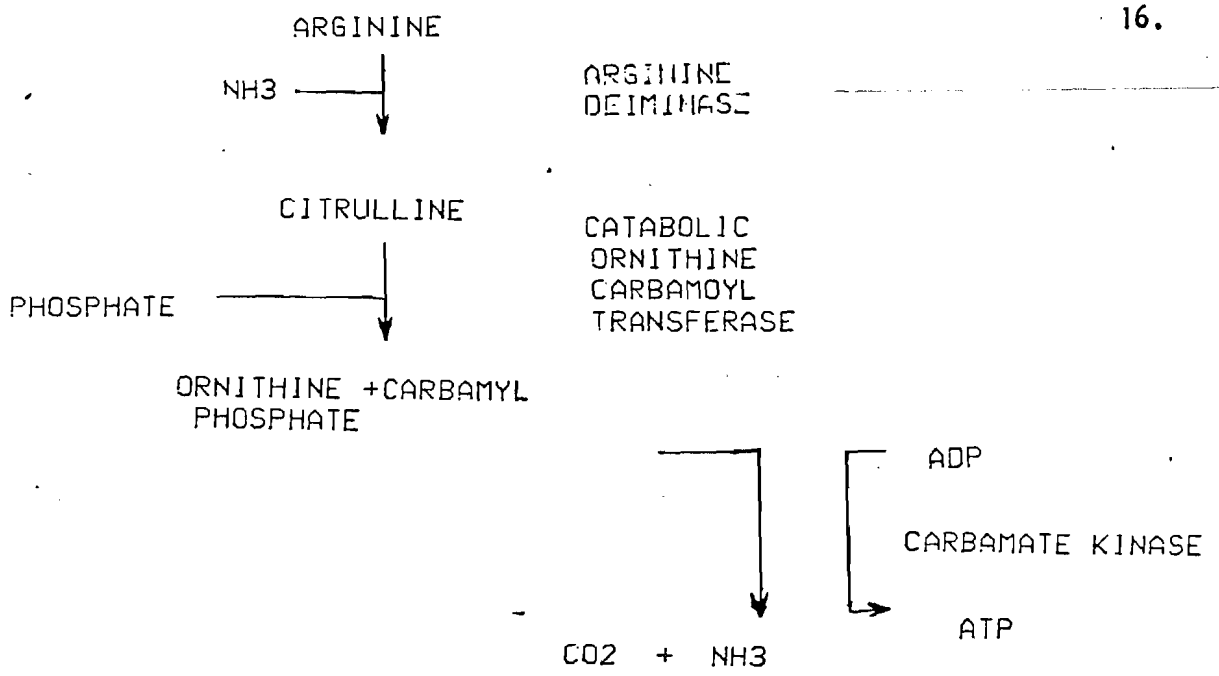


Fig. 4a ARGININE CATABOLIC PATHWAY IN P. aeruginosa.

(STALON et. al. 1967; 1972)

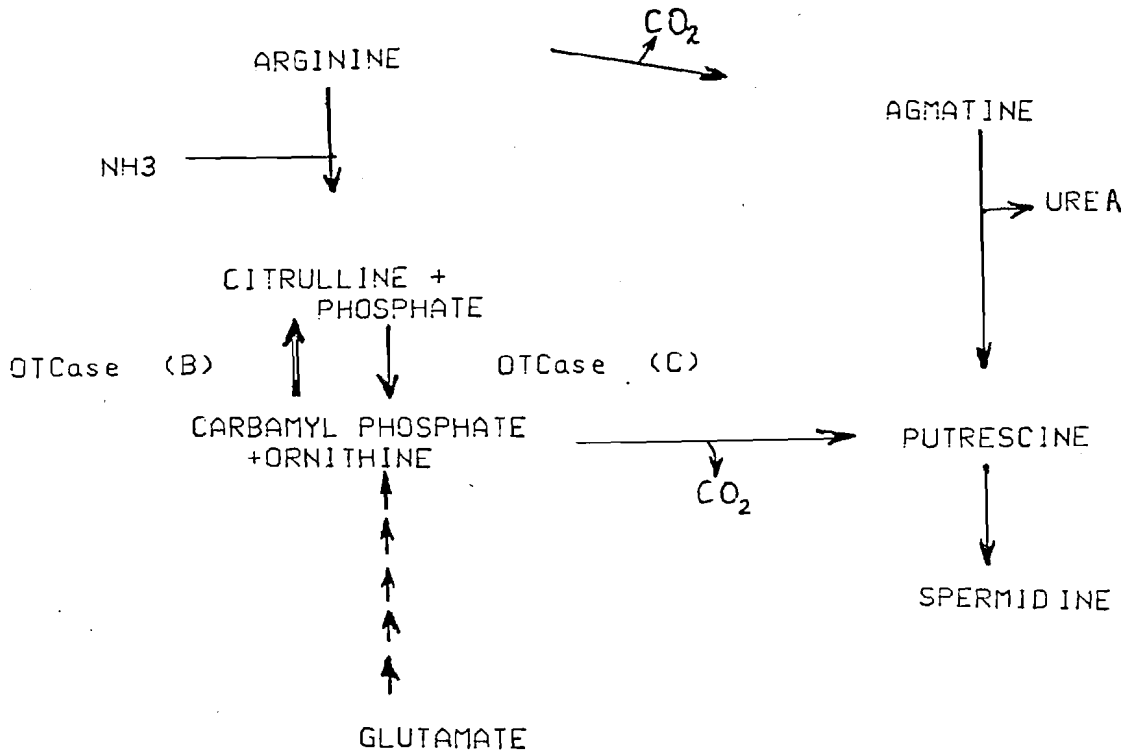


Figure 4b..Interaction of arginine Biosynthetic & Catabolic Pathways in *P.aeruginosa*.

(Stalon *et al.*, 1972)

programme would be one unable to utilise arginine. As already found *argF* product is the only enzyme under significant repressor control. Hence, it is expected that subcloning of the gene *argF* from the R prime plasmid, pCR1 into a multicopy vector such as pAT153 or pBR322 and ultimately introducing stably into a Arg^- host organism would mop up the *arg* repressor and allow overproduction of arginine. In addition to achieving this ultimate goal this study is expected to throw light on the regulation mechanism of the amino acid biosynthesis in *Pseudomonas aeruginosa* which is not fully known unlike in the case of *E.coli*.

CHAPTER 2. - MATERIALS AND METHODS

(i) Organisms and plasmids used

(a) The bacterial strains and the plasmids used in this study are listed in Table I with their relevent genotypes and phenotypes.

Table 1. Bacterial strains and plasmids.

Strain/plasmid	Genotype and/phenotype	Source/Reference
Pseudomonas putida		
PPN 1092 (pCRI)	argF407, met406, trpF411	A.F. Morgan
Escherichia coli		
HB101	Tet^S, Amp^S, Sm^R	Bolivar and Backman (1979)
W4100 Arg	argF, argI, thi, Tet^S, Amp^S	R.W. Hedges
Plasmids		
pCRI	Tc^S, Cb^R, Km^R, *argF/G*$^+$, *amiER*$^-$	Rice (1984)
Vectors		
pBR322	Tet^R, Amp^R	Appendix I & II
pAT153	Tet^R, Amp^R	Appendix I & II

(b) Maintenance

All strains in constant use were maintained on L-Broth Agar (LBA) slopes in Universal bottles at room temperature. R prime plasmid, pCRI containing strain *P.putida* PPN1092 was maintained on *Pseudomonas* selective minimal agar slopes containing 500 $\mu g.ml^{-1}$ carbenicillin. *E.coli* strains carrying the pAT153, pBR322 and the recombinant pAT153 were maintained on LB-agar slopes containing selective antibiotics such as ampicillin (50 $\mu g.ml^{-1}$) or tetracycline (25 $\mu g.ml^{-1}$).

(ii) Chemicals and Reagents

All the chemicals used in this study were either of laboratory grade or AR grade (for reagents and buffers) bought from Standard Companies. Antibiotics, ampicillin, tetracycline and carbenicillin were also obtained from Standard manufacturers/

(iii) Growth Media

(a) L-Broth

	Per liter
Bacto tryptone	10g
Bacto yeast extract	5g
NaCl	6g

(b) L-Broth Agar

The above medium containing 1.2% (w/v) Difco agar. This was used for plates and slopes.

(c) Pseudomonas minimal media

The composition of basal medium was as follows:

	Per liter
K_2HPO_4	12.5g
KH_2PO_4	3.8g
$MgSO_4\ 7H_2O$	0.5g
Trace element solution	5 ml

Composition of trace element solution:

$FeSO_4(NH_4)SO_4\ 6H_2O$	116mg
H_3BO_3	232mg
$CoSO_4\ 7H_2O$	96mg
$CuSO_4\ 5H_2O$	8mg
$MnSO_4\ 4H_2O$	8mg
$(NH_4)_6Mo_7O_{24}\ 4H_2O$	22mg
$ZnSO_4\ 7H_2O$	174mg

$(NH_4)_2\ SO_4$ at a concentration of $1g.l^{-1}$ was added to the above medium as nitrogen source. Sodium succinate at $10g.l^{-1}$ was added as a carbon source. Besides these, separately sterilized tryptophan and methionine were added to the medium to a final concentration of $30\mu g.ml^{-1}$ for growing *P.putida* PPN1092. The medium was adjusted to pH 7.2.

(d) *Pseudomonas* minimal agar

1.2% agar in the above medium.

(e) *E. coli* minimal media (MG9)

	Per liter
Na_2HPO_4	6g
$KH_2\ PO_4$	3g
NH_4Cl	1g
NaCl	0.5g

pH adjusted to 7.4, autoclaved and then added:

1M $MgSO_4$	2ml
20% glucose	10ml
1M $CaCl_2$	0.1ml

The above solutions were separately autoclaved.

(f) MG¥9 Agar

The above medium containing 1.2% agar.

(iv) Growth of bacteria

(a) Growth in liquid media

Bacterial strains were grown in liquid media in amounts ranging from 2ml to 100ml. Volumes of cultures below 10ml were grown in Universal bottles with reciprocal shaking in a water bath. Larger volumes of cultures were grown in concial flasks of suitable sizes e.g., 50ml in 500ml flasks, 100ml in 1 liter flasks etc. on a rotary shaker. *E. coli* strains were incubated at 37°C and *P. putida* at 29°C.

(b) Growth on Solid Media

Volumes of 0.1 or 0.2ml of cell suspensions were plated on appropriate (selective) media and spread uniformerly with a sterile glass spreader. The plates were incubated overnight at appropriate temperatures.

(v) Plasmid isolation and analysis

(a) Large scale extraction of plasmid DNA from *P. putida*

This was done according to Wheatcroft and Williams (1981) method as modified by Rice (1984).

P. putida strain PPN 1092 was grown to stationary phase in 100ml of selective minimal media containing 500µg.ml^{-1} carbenicillin. The cells were collected by centrifugation at 4000 rpm for 10 min. The cells were then resuspended in 1.6ml of Reagent A and 0.4ml Reagent B was added and then the tubes were shaken until the colour turned from blue to green. Then the samples were vortexed vigorously for 5 min.

The suspensions thus prepared were layered onto 19.5ml sucrose gradients in 25ml thinwalled polycarbonate centrifuge tubes. This was then centrifuged at 24,000 rpm for 3 hr. at 20°C in a swing out rotor.

After removing from the rotor the tubes were fixed on a clamp and pierced by a hypodermic needle at a point about 1-1.5cm above the bottom. Fractions of 10 drops (about 1ml) were collected in Eppendorff tubes. The fractions were then tested by running on agarose gel to ascertain which fractions had the purest and most concentrated plasmid DNA. These fractions were used for further studies.

(b) Rapid plasmid preparations

This was also done according to Wheatcroft and Williams (1981) method. 10ml culture *P. putida* 1092 grown to stationary phase in 20ml universal bottles were centrifuged and the cell pellet was resuspended in 1ml of sterile water. After transferring to an Eppendorf tube this was again spun

at 4000 rpm for 10 min. To the pellet 100µl Reagent A was added and mixed well followed by the addition of 25µl of Reagent B. The tube was then inverted about 20 times in 1 min. during which time the colour changed from blue to green. The sample was then vortexed for 2 to 3 min. 20µl of this sample was loaded onto the gel and electrophoresed.

This method also tried for detection of plasmids in *E. coli* transformant strains.

Reagent A:

TE buffer containing 5% Dow Corning Antifoam RD emulsion

0.1mg xylene cyanol FF.ml^{-1}

TE buffer (pH 8.0)

Tris/HCl 10mM (pH 8.0)

Na_2 EDTA 1mM (pH 8.0)

Reagent B:

1M NaOH saturated at 20°C with sodium dodecyl sulphate

Preparation of Sucrose Gradients:

Sucrose gradients were prepared by the method described by Baxter-Gabbard (1972). A 20% (w/v) sterile solution of sucrose was slowly frozen and then thawed in centrifuged tubes to form sucrose gradients. These were again frozen and stored at -20°C and thawed slowly before use.

(vi) Rapid extraction of plasmid DNA from *E. coli* cells

The method described by Holmes and Quigley (1981) was used with slight modifications for rapid preparation of plasmids from *E. coli* strains.

5 or 10ml of overnight broth culture was centrifuged at 4000 rpm for 10 min. and the pellet was resuspended in 600µl of STET buffer. This was transferred to an Eppendorf tube and 40µl of lysozyme solution (10mg/ml) was added and mixed gently by inverting the tube 5 times. This was left at room temperature for about 15 min. and then the tubes were transferred to a

boiling water bath where they were kept for exactly 40 seconds. Immediately after this the sample was centrifuged at 15,000 rpm for 10 min. at room temperature. The pellet formed was removed carefully by dragging it up through the side of the tube using a toothpick. To the supernatant was added an equal volume of isopropanol and kept at -20°C for at least 10 min. before centrifuging at a speed of 15,000 rpm for 10 min. at 4°C. All the liquid was removed from the tube without disturbing the DNA pellet. The tube was kept inverted on a tissue paper to drain for 5 min. To the DNA pellet was then added 50µl of TE buffer (pH 8.0) and left at room temperature for at least 10 min. 10-15µl of this preparation (to which loading dye was added) was loaded onto the gel for electrophoretic analysis.

STET Buffer (pH 8.0)

Sucrose	8%
Triton X-100	0.5%
Na_2 EDTA (pH 8.0)	50mM
Tris HCl (pH 8.0	10mM

(vii) Gel electrophoresis

DNA samples were examined on horizontal gels containing 0.7% (w/v) electrophoresis grade agarose. For analysis of large plasmids electrophoresis was carried out overnight using large gels and was run at 80V. For detecting miniplasmids prepared by rapid method and restricting endonuclease digests the electrophoresis was carried out at 80V for 1½ hrs. on mini gels. The gels were prepared and run in TBE buffer. The gels were stained in 1µg ethidium bromide ml^{-1} solution and photographed on a UV transilluminator using a polaroid camera and an X7 red filter.

Tris-Borate (TBE) Buffer

A stock solution of 5 times concentrated was diluted prior to use. The diluted buffer (working solution) consisted of 89mM Tris base, 89mM boric acid and 2.5mM Na_2 EDTA (pH 8.0).

(viii) Restriction endonuclease digestion

Buffers used for restriction enzyme digestion were those recommended by the manufacturers.

The reaction was carried out in Eppendorf tubes at 37°C for 2-4 hrs. with appropriate quantity of the respective enzymes. On completion of the reaction aliquotes of digests were taken and analysed electrophoretically. The remaining restricted plasmid DNA was precipitated by adding 3M sodium acetate (1/10 of the volume of DNA solution) and absolute alcohol (2.5 times the volume of DNA solution) and keeping at -70°C for 30 min. The precipitate was centrifuged at a speed of 15,000 rpm at 4°C. After draining and drying, the pellet was resuspended in ligation buffer (in the case of pCR1 digest) or in STE buffer (in the case of vector plasmids).

Ligation Buffer

Tris HCl (pH 7.8)	66mM
$MgCl_2$	5mM
dithiothreitol (DTT)	5mM
ATP	1mM

STE Buffer

Tris HCl (pH 8.0)	10mM
NaCl	100mM
Na_2EDTA (pH 8.0)	1mM

(ix) Dephosphorylation of the restricted vector DNA

The restricted vector DNA (pBR322 and pAT153) were treated with calf intestinal alkaline phosphatase to prevent them from self-ligation during ligation with DNA fragments of pCR1. To the DNA solution in STE buffer appropriate amounts of the enzyme was added as specified by the manufacturers and incubated at 37°C for 1hr. After the reaction was complete the DNA was precipitated by adding sodium acetate and ethanol and keeping at -70°C for

30 min. The precipitate was then pelleted by centrifugation, drained and dried. This was then redissolved in ligation buffer.

(x) Ligation

Solutions of vector DNA and the DNA fragments of the R prime plasmid, pCR1 (both solutions contained approximately 1µg DNA) in ligation buffer were mixed and aliquotes were taken for electrophoretic analysis. Then 1µl of T_4-DNA ligase was added, mixed well and incubated at 8°C for 24hrs. These samples were used for transforming the competent cells of *E. coli* strains.

(xi) Preparation of Competent Cells

Competent cells of *E. coli* strain HB101 as well as W4100Arg were prepared as follows:

50ml of prewarmed (37°C) L-broth taken in 500ml conical flask was inoculated with 1ml of overnight culture in the same broth (grown in 20ml universal bottle containing 5ml medium with shaking). Incubated at 37°C with vigorous shaking until the OD_{450} was 0.5-0.6. The the culture was chilled in an ice bath for 10 min. before it was centrifuged at 4°C and at a speed of 4000 rpm for 10 min. The cell pellet was resuspended in 25ml of chilled 0.1M $MgCl_2$. Centrifuged again at 4°C. The cell pellet was then resuspended in 2.8ml 0.1M $CaCl_2$ and kept in an ice bath for 60 min. Now the cells were competent.

(xii) Transformation of *E. coli* strains

200µl of the competent cells were mixed thoroughly with the ligated DNA sample which was made up to 100µl in SSC/$CaCl_2$ mixture and kept in ice bath for 45 min. Heat shocked at 37°C for 5 min. and again kept in ice for 30 min. more at the end of which 700µl prewarmed (37°C) L-broth was added and incubated at 37°C for 90 min. without shaking.

100-200µl of this cell suspension was spread on Petri plates containing respective media for selecting the transformants. For total counts 100µl of appropriately diluted samples (usually 10^{-5} - 10^{-8}) were plated. The plates were kept inverted for incubation at 37°C.

SSC (Standard Saline Citrate)/$CaCl_2$ mixture consisted of 0.15M sodium chloride, 0.015M trisodium citrate mixed at pH 7.0. 0.1M $CaCl_2$ was mixed with the above mixture in the ratio 4:3 just before use and preserved on ice for use.

CHAPTER 3 - RESULTS

(i) Plasmid DNA preparation and Analysis

The R prime plasmid, pCR1 DNA from *P. putida* PPN 1092 was prepared according to a modified Wheatcroft and Williams (1981) method. The method was found to be quite suitable for large scale preparation of pCR1 plasmid DNA, though the purity of the plasmid fractions were found to vary from preparation to preparation. Fig. 5a and b show the agarose gel electrophoretic pattern of different fractions obtained from the sucrose gradient. Fractions 5,6,7 and 8 (Fig 5a and 5b) were found to contain reasonably pure, though fairly dilute plasmid DNA. However, these fractions were quite sufficient for restriction enzyme digestion and analysis.

Attempts to concentrate these plasmid preparations by sodium acetate ethanol precipitation did not yield the expected results: the plasmid DNA was lost during concentration. Hence, for restriction analysis the original dilute fractions were used. Only after the digestion was the DNA concentrated by acetate-ethanol method. Fig. 6 shows the gel electrophoresis patterns of the plasmid DNA digested with Bam H1 prior to and subsequently concentrated. The size of the plasmid DNA after concentration did not compare with either the intact plasmid or the contaminating chromosomal DNA. The complete digest of this DNA showed only 4 bands whereas the digest of intact plasmid (~~not shown in the figure~~) showed large number of bands (the size of which varied) from approximately 226Kb down to less than 2Kb (Fig. 6). In Figs. 7a and b are shown the gel patterns of PstI and HindIII digests of pCR1 along with pAT153 plasmid.

(ii) Recombination of pCR1 fragments with pAT153 or pBR322

The R prime plasmid pCR1 was digested with BamH1 and Pst1 separately. They were mixed with similarly cut vector DNAs, pAT153 and pBR322 (Appendix 1) i.e. BamH1 cut pCR1 fragments were mixed with BamH1 cut pAT153 or pBR322 and

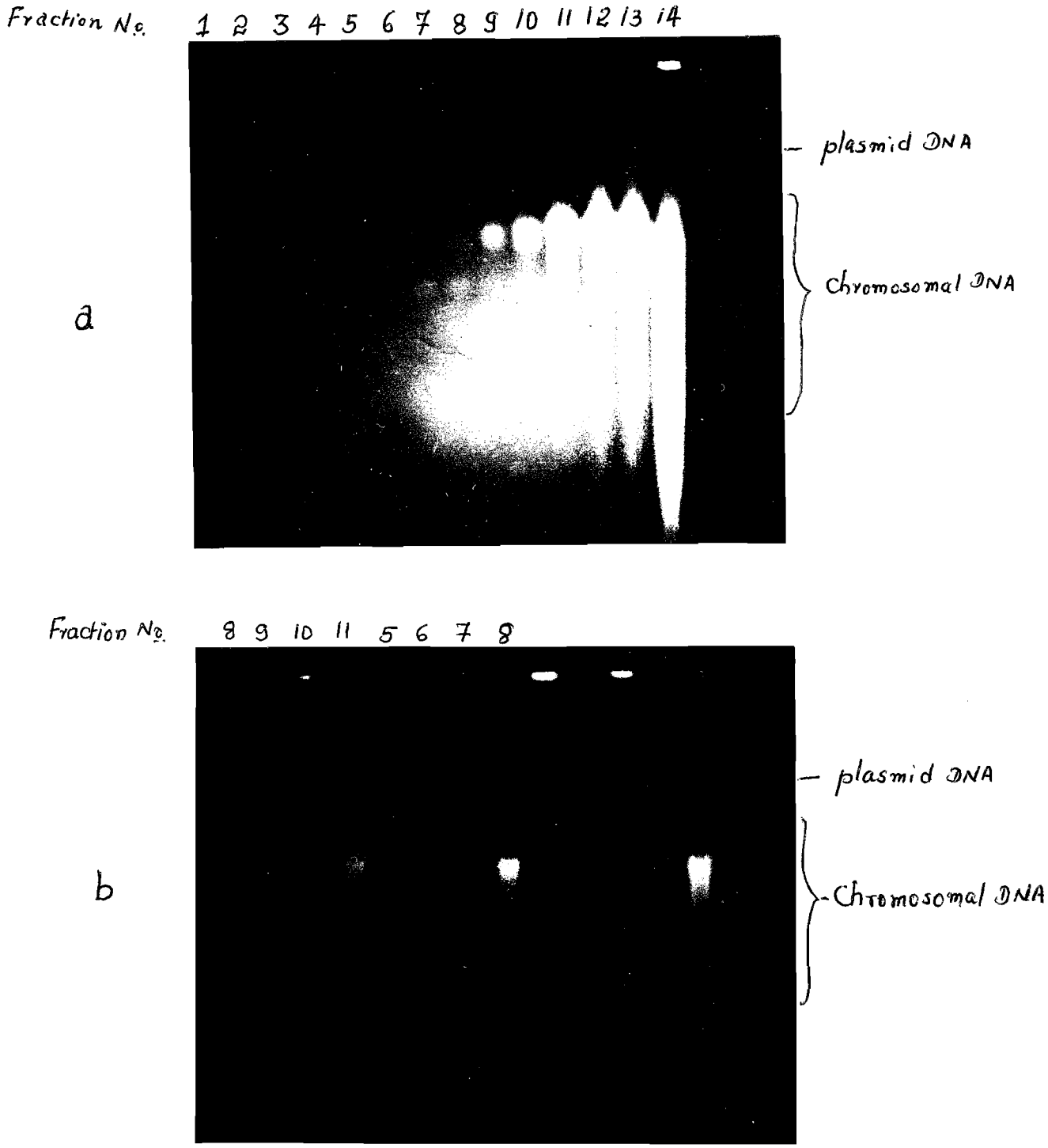


Fig. 5a & b: R prime plasmid pCR1 fractions from Sucrose gradient (from different batches)

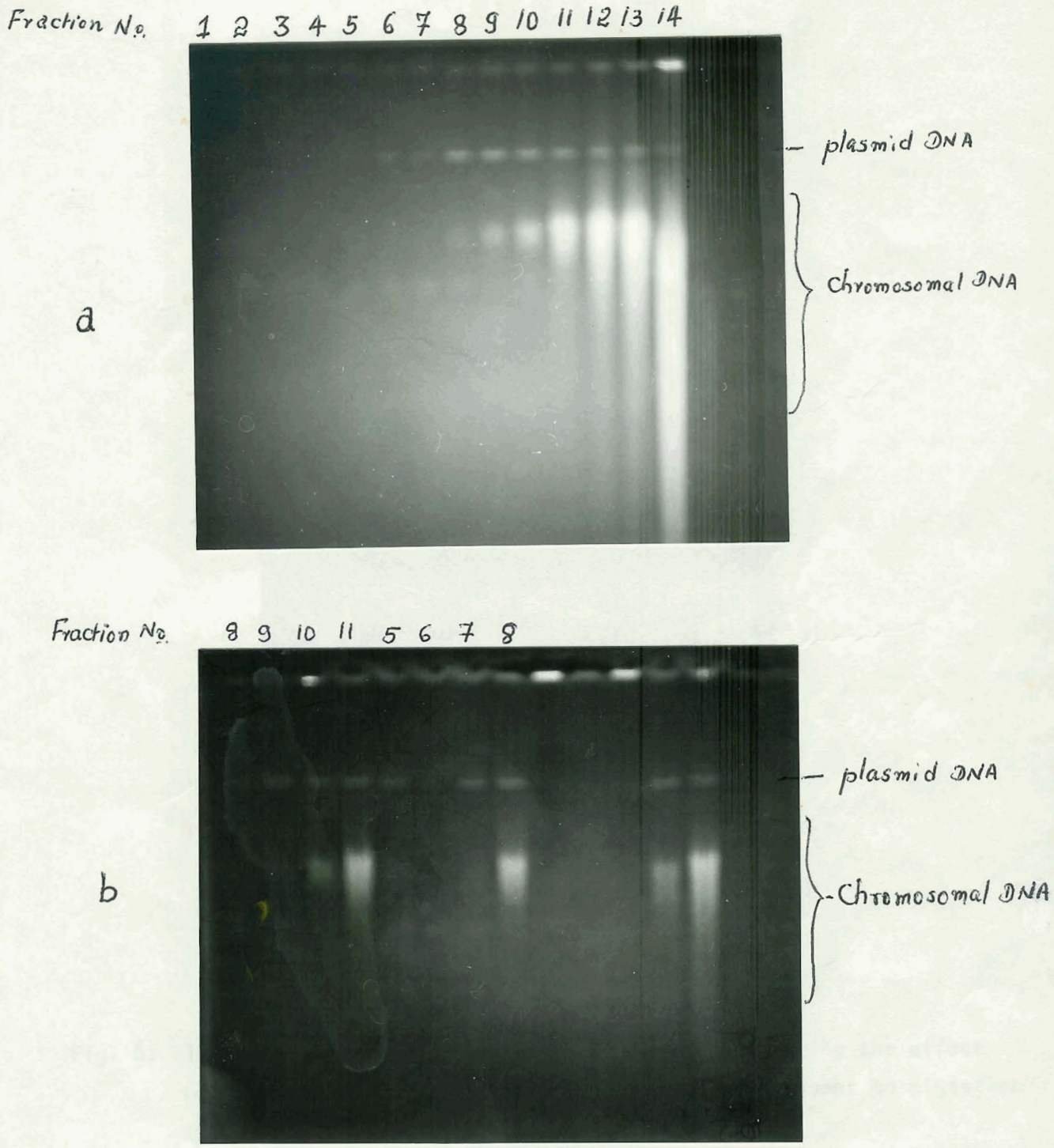


Fig. 5a & b: R prime plasmid pCR1 fractions from Sucrose gradient (from different batches)

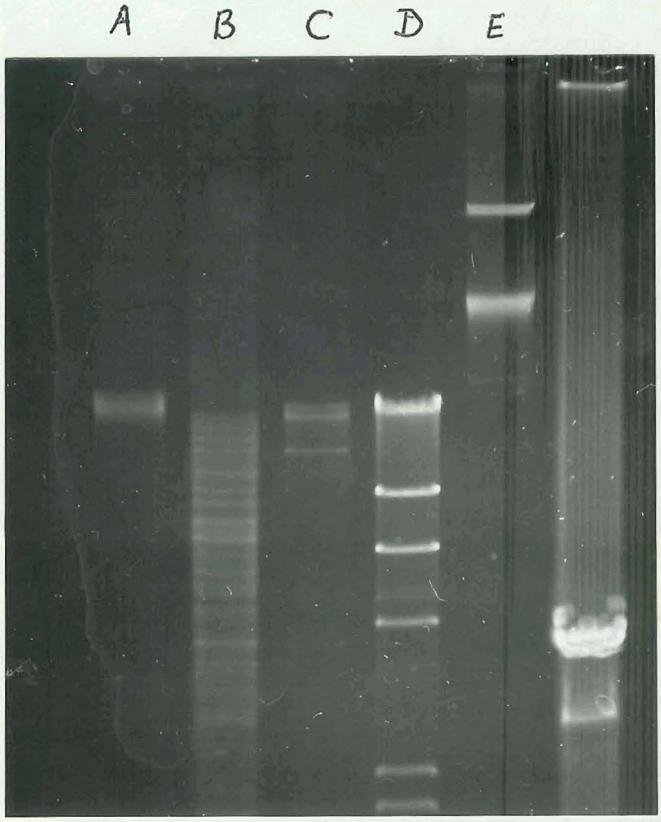


Fig. 6: The gel pattern of the BamH1 digests of pCR1 showing the effect of concentrations of the plasmid prior and subsequent to digestion with BamH1

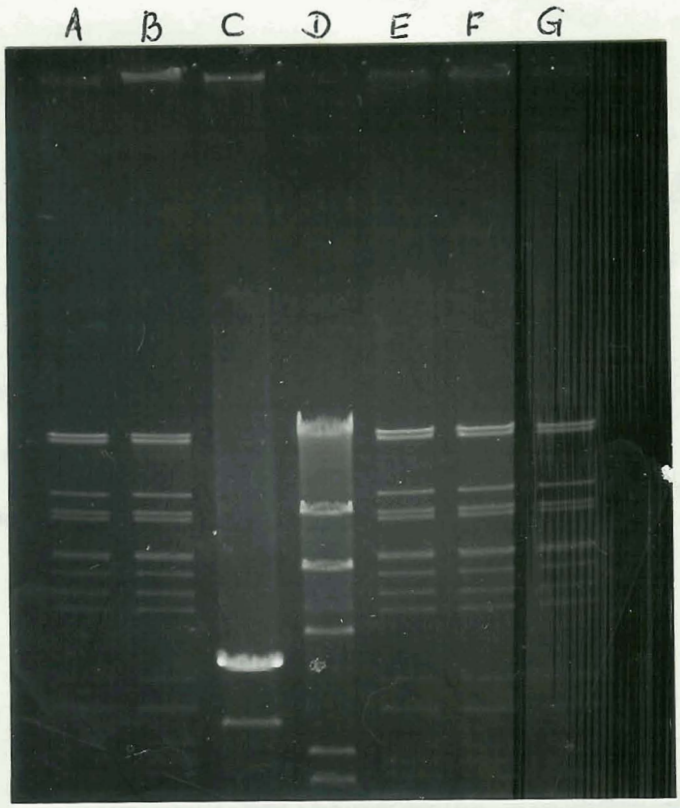
A - The plasmid preparation after concentration with acetate-ethanol precipitation

B - BamH1 digest of pCR1 (done prior to concentrations)

C - BamH1 digest of acetate-ethanol precipitate of the plasmid

D - HindIII digest of Lambda DNA

E - Fraction of plasmid preparation having contaminating chromosomal DNA (for comparison with the concentrated preparation)

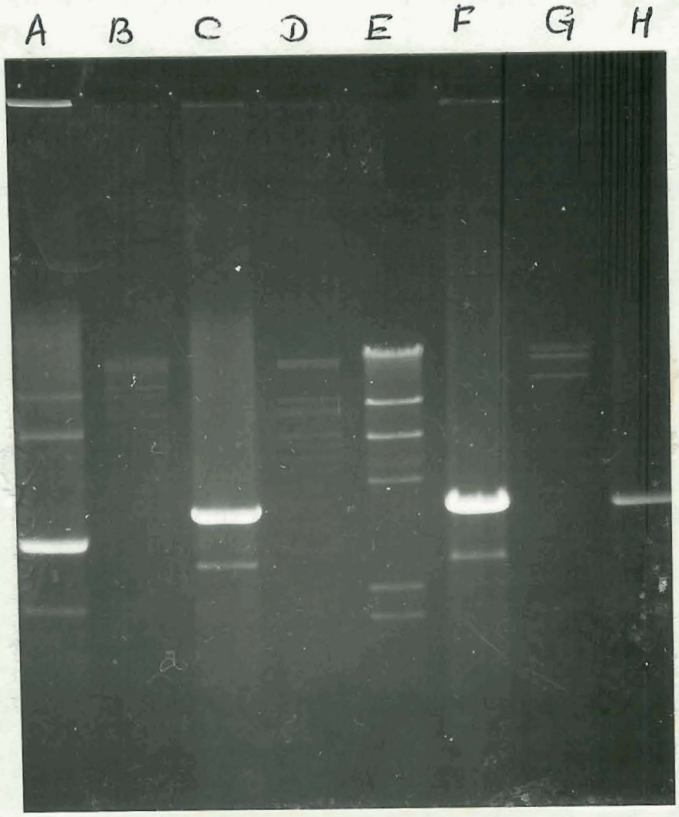


a

A, B., E, F & G - Restriction digests of pCR1 with Pst 1

C - Pst I cut pAT153

D - Hind III digest of λ DNA.



b

A. whole plasmid pAT153

B. Hind III digest of pCR1

C. Pst I cut pAT153.

D. Pst I digest of pCR1

E. Hind III digest of λ DNA.

F & H. BamHI Cut pAT153

G. Bam HI digest of pCR1. (after Concentrating the plasmid)

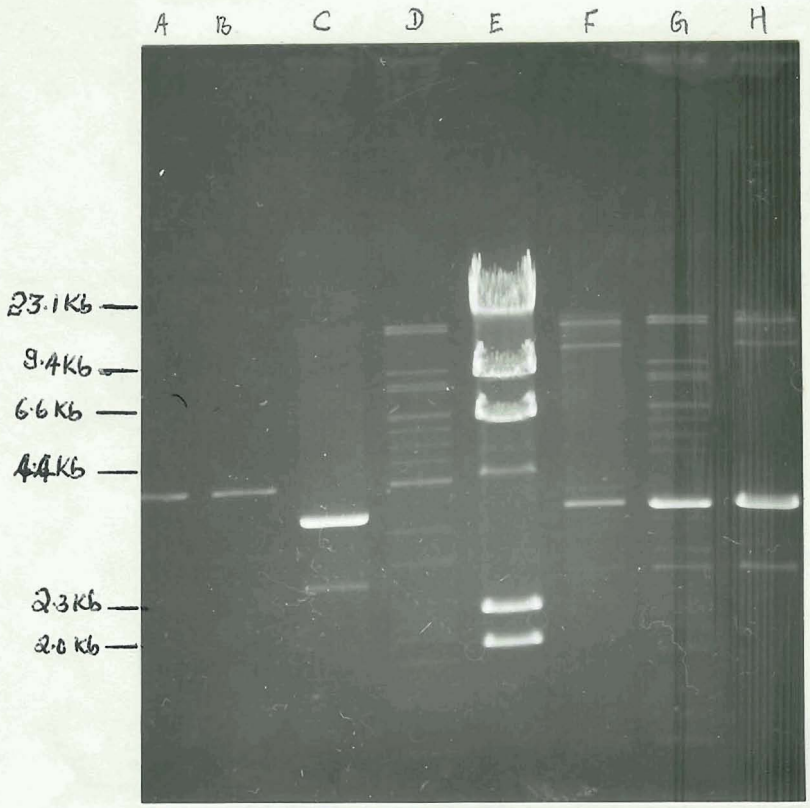
Fig. 7a & b: Restriction enzyme digests pCR1 preparations (fractions from sucrose gradient)

Pst1 cut pCR1 fragments with Pst1 cut pAT153 or pBR322 and ligated. The restriction enzyme cut vector DNAs were treated with alkaline phosphatase prior to ligation. In Fig. 8 are shown the gel patterns of the DNA mixture of pCR1 fragments and restricted vectors before the beginning of ligation. It could be seen that fairly good ligation has occured in the case of pAT153 both in BamH1 and Pst1 cut samples whereas the ligation was poor in the case of pBR322. The approximate size of the ligated DNA in the case of Pst1 cut and BamH1 cut samples were 3.9-4.5Kb and 3.8Kb respectively (Figs. 10 and 11).

(iii) Transformation of *E. coli* HB101

Attempts to transform *E. coli* W4100 Arg$^-$ with *in vitro* constructed recombinant DNA (recombinant pAT153 carrying fragments of pCR1 DNA) was not successful. Even whole pAT153 as well as pBR322 failed to transform this strain. Hence, it was decided to transform other strains of *E. coli* such as HB101 which could be easily transformed and extract plasmids from the recombinant strains which then could be used for transforming *E. coli* W4100 Arg$^-$.

Competent cells of *E. coli* HB101 were prepared and transformed with DNA samples. The DNA samples used were pAT153 to which fragments of pCR1 were inserted and ligated at Pst1 site and BamH1 site and pBR322 in which pCR1 DNA fragments were inserted at Pst1 site. Whole plasmid preparations of pAT153 as well as pBR322 were also used for transformations as control. The cells treated with recombinant plasmids with inserts at Pst1 site were plated on L-broth agar plates containing 25μg.ml^{-1} tetracycline and those treated with plasmids carrying inserts at BamH1 site were plated on L-broth agar containing 50μg.ml^{-1} ampicillin without dilution. The results are given in Table 2. As could be seen from the table pBR322 restricted with Pst1 and ligated with fragments of pCR1 similarly restricted failed to transform the cells whereas the whole plasmid (pBR322) did transform the cells with fairly



A: PstI Cut pBR322

B: BamHI pBR322

C: PstI Cut pAT153

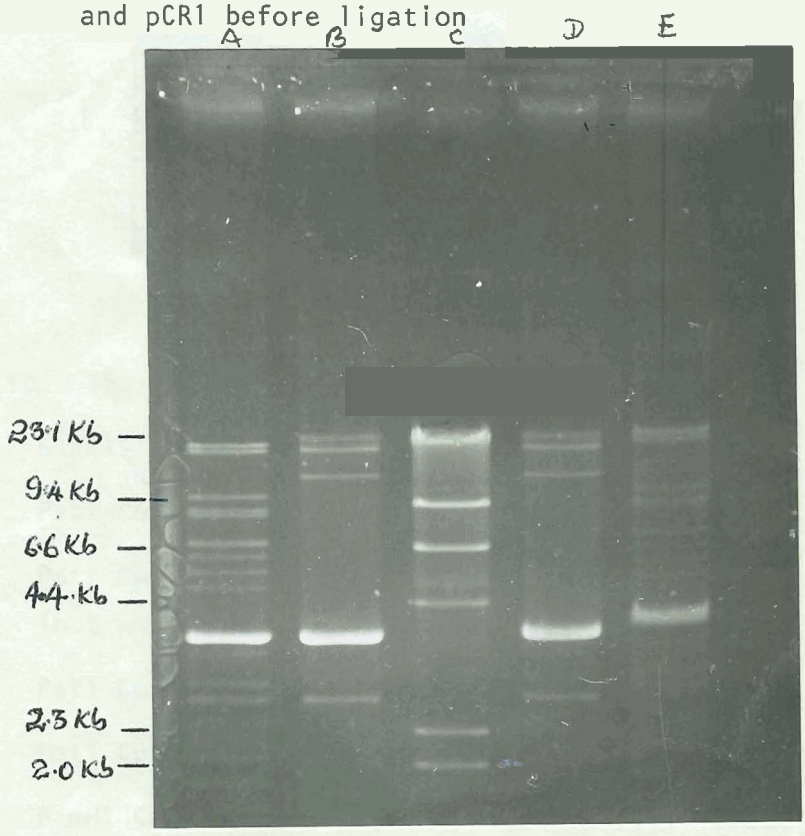
D: Mixture of PstI Cut pBR322 and pCR1

E: HindIII digest of λ DNA

F & H: Mixture of BamHI Cut pAT153 and pCR1

G: Mixture of PstI cut pAT153 and pCR1

Fig. 8: Mixtures of restriction enzyme digested vector DNA (pAT153 and pBR322) and pCR1 before ligation



A: Mixture of PstI Cut pAT153 and pCR1

B: Mixture of BamHI Cut pAT153 and pCR1

C: HindIII digest of λ DNA

D: BamHI Cut DNA mixture after ligation (not well ligated)

E: PstI Cut DNA mixture after ligation (ligated fairly well)

Fig. 9: Mixtures of restriction enzyme digested pAT153 and pCR1 (similarly digested) before and after ligation reaction

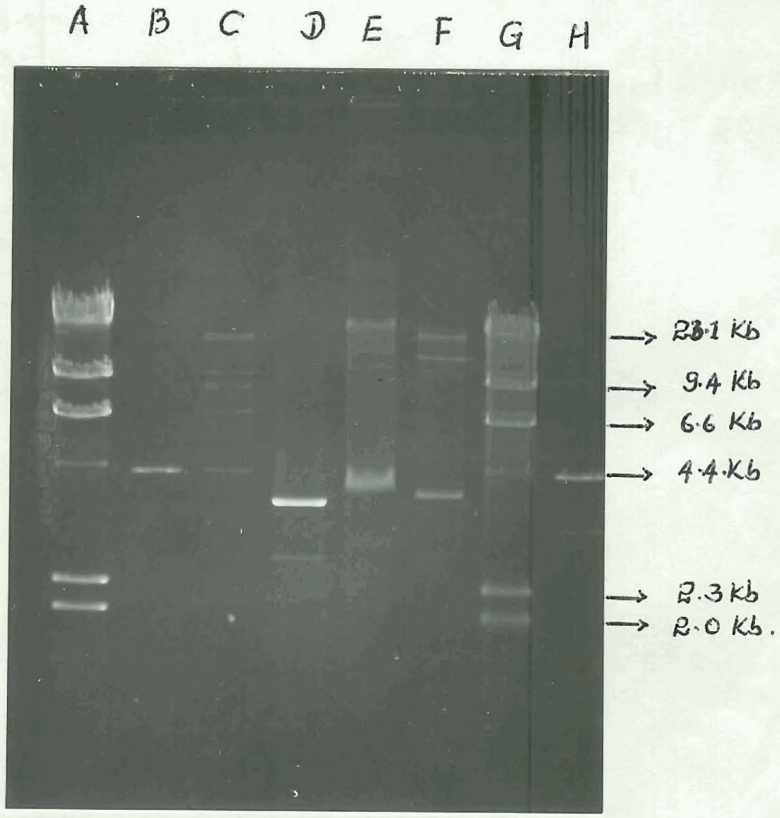


Fig 10: The ligated vector DNA and pCR1 fragments

A. HindIII digest of λDNA

B. Pst1 Cut pBR322

C. Pst1 Cut pBR322 and pCR1 fragments after ligation reaction (not well ligated)

D. Pst1 Cut pAT153

E. Pst1 Cut pAT153 and pCR1 fragments after ligation (well ligated)

F. BamH1 Cut pAT153 and pCR1 fragments after ligation (well ligated)

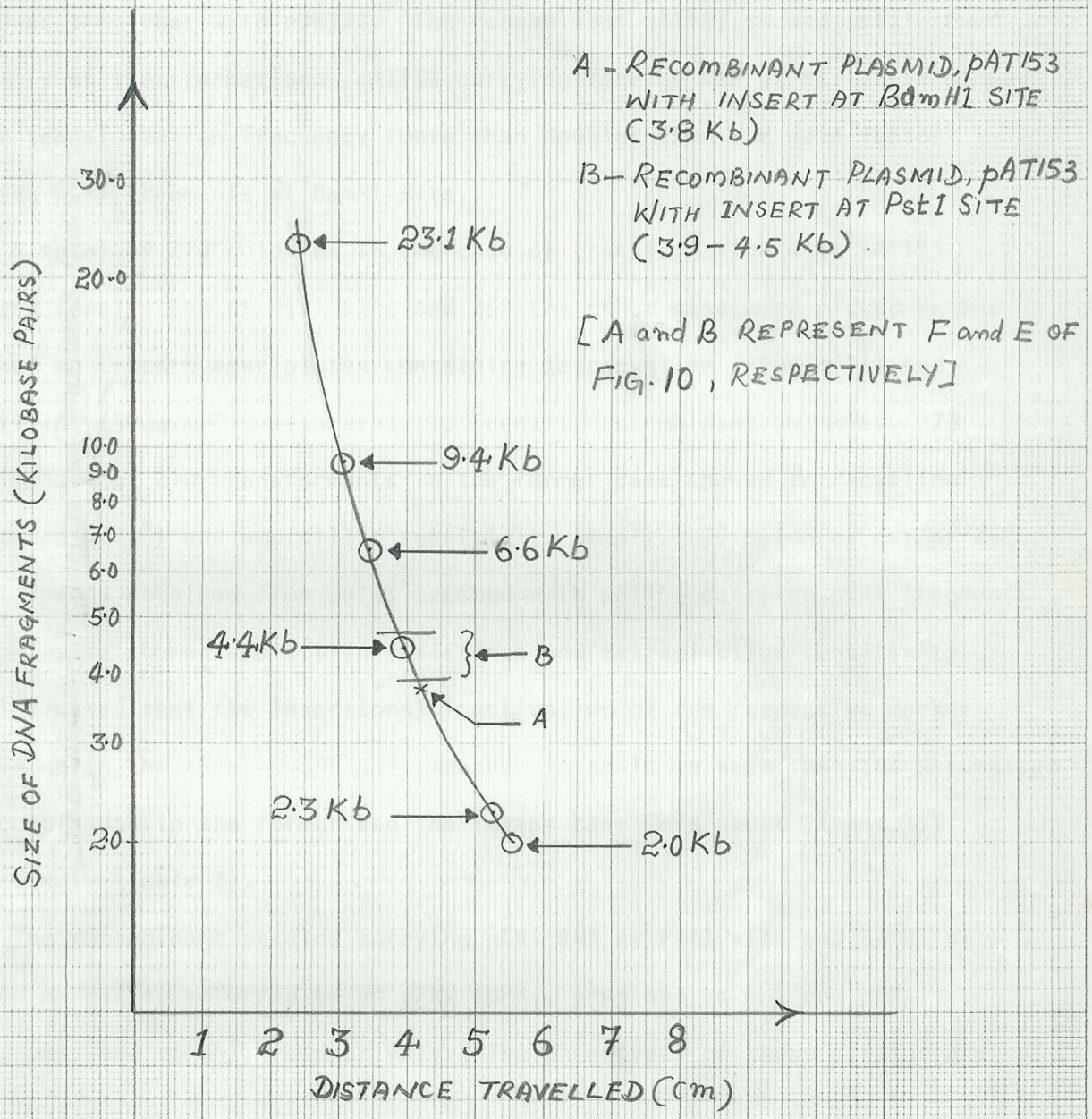


FIG 11 SIZE DETERMINATION OF RECOMBINANT PLASMID, pAT153 USING Hind III DIGEST OF LAMBDA DNA AS STANDARD.

high frequency. The frequency of transformation by pAT153 whole plasmid was lower than that with pBR322. The recombinant pAT153 showed still lower frequency of transformation. pAT153 carrying foreign DNA at Pst1 site showed higher transformation frequency (more than double) than the same vector carrying pCR1 fragments at BamH1 site.

A total of 210 colonies in the case of transformation with pAT153 carrying foreign DNA at Pst1 site and 252 the other case were picked up and streaked on L-broth agar plates containing tetracycline ($25\mu g.ml^{-1}$) and ampicillin ($50\mu g.ml^{-1}$) separately to identify recombinant colonies. 73 colonies out of 210 transformants in the former case showed tetracycline resistance (Tet^R) and ampicillin sensitivity (Amp^S). 78 colonies out of 252 transformants obtained from cells treated with pAT153 carrying pCR1 fragments of BamH1 site showed ampicillin resistance and tetraclycline sensitivity. This indicated that the insertional inactivation of the respective marker gene in which the foreign DNA was cloned. It could be seen that the percentage of recombinants in the former and the latter case were about 31 and 35% respectively (Table 2).

The recombinant strains carrying pCR1 DNA at Pst1 site and BamH1 site will be hereafter referred to as pPK_n (pPK_1, pPK_2 and so on) and pBK_n (pBK_1, pBK_2 and so on), respectively. The phenotypes of these strains are as follows:

1. pPK_{1-n} - Tet^R, Amp^S
2. pBK_{1-n} - Amp^R, Tet^S

(iv) Isolation of plasmids from recombinant strains by rapid plasmid isolation method

The above recombinant strains were grown in L-broth containing the appropriate antibiotic overnight, pooled as given in Table 3, and the plasmid DNA was extracted by a slightly modified rapid plasmid extraction method of

Table 2. Transformation of *E.coli* HB101 with plasmid vectors, pAT153 and pBR322 carrying pCR1 fragments at PstI and BamHI sites and also with whole pAT153 and pBR322 plasmids

Plasmid vector/site of insertion of pCR1 fragments	No.of transformants (per ml.of cell suspension)	Transformation frequency (%)*	Percentage of recombinants
1. pBR322 - whole plasmid	1.44×10^{4}	3.9×10^{-4}	-
2. pBR322/PstI	nil	-	-
3. pAT153 - whole plasmid	3.91×10^{2}	1.06×10^{-5}	-
4. pAT153/PstI	2.8×10^{2}	7.56×10^{-6}	34.76
5. pAT153/BamHI	1.2×10^{2}	3.24×10^{-6}	30.95

*The suspension of competent cells conatined 3.7×10^{9} cells ml^{-1}.

Holmes and Quigley (1981).

Table 3: The pooling of recombinant plasmid preparations

Pooled Plasmid Preparation	From Strain No.	Pooled Plasmid Preparation	From Strain No.
PP-1	pPK_1 - pPK_{13}	A	pBK_1-pBK_{10}
PP-2	pPK_{14} - pPK_{26}	B	pBK_{11} - pBK_{20}
PP-3	pPK_{27} - pPK_{38}	C	pBK_{21} - pBK_{30}
PP-4	pPK_{39} - pPK_{47}	D	pBK_{31} - pBK_{40}
PP-5	pPK_{48} - pPK_{56}	E	pBK_{41} - pBK_{50}
		F	pBK_{51} - pBK_{60}
PP-6	pPK_{57} - pPK_{66}	G	pBK_{61} - pBK_{70}
PP-7	pPK_{67} - pPK_{73}	H	pBK_{71} - pBK_{78}

Fig. 12 shows the gel pattern of pooled plasmid preparations PP-1 through PP-7. As could be seen the size of the plasmids ranged from approximately 3Kb to as big as about 100Kb.

The same method was used for isolation of plasmids pAT153 and pBR322 from *E. coli* possessing these plasmids. The preparations were found to be reasonably free from chromosomal DNA.

(v) Transformation of *E. coli* W4100 Arg Strain

As already mentioned *E. coli* W4100 Arg-strain is an arginine requiring strains which has also no resistance to either ampicillin or tetracycline. The competent cells of this strain were prepared according to the same procedure as was used in the case of *E. coli* HB101.

Transformation of the competent cells were carried out with batches of pooled plasmid DNA preparations (Table 4). The different batches of pooled plasmid DNA contained further combinations of pools of plasmids

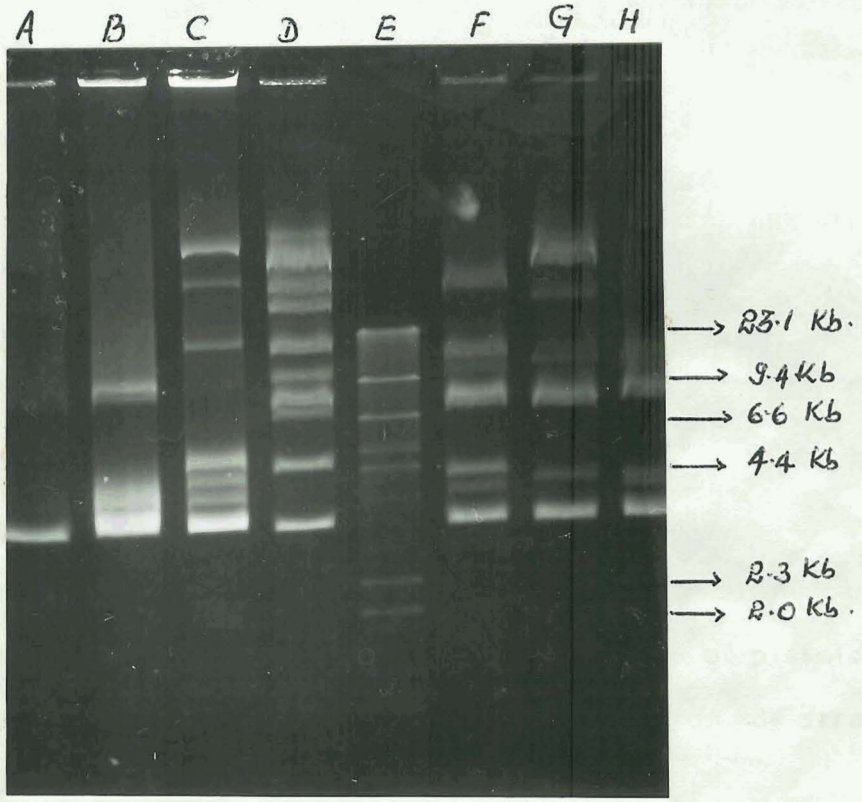


Fig 12: Recombinant plasmid pools from transformant pPK strains

A,B,C,D,F,G, and H represent plasmid pools PP-1, PP-2, PP-3, PP-4, PP-5, PP-6 and PP-7 respectively (Table 3)

E: HindIII digest of λ DNA

given in Table 3.

Table 4: Batches of plasmid DNA from different pools

Batch	Plasmid Pool and Source	
I	PP-1, PP-2 and PP-3	pPK strains
II	PP-4 and PP-5	
III	PP-6 and PP-7	
IV	A and B	pBK strains
V	C and D	
VI	E and F	
VII	G and H	

The selective media used was *E. coli* minimal agar (MG19 Agar), L-broth agar containing ampicillin and tetracycline separately.

The results of transformation are given in Table 5. As can be seen in the table there were no transformants in the case of plasmids from pPK strains, whereas all the batches of plasmid pools from pBK strains showed transformation with varying frequencies.

The transformant colonies showed slow growth on minimal medium (MG9 agar). Though minute colonies were visible on the plate after about 20 hrs. incubation it took three days to show reasonably good growth. The size of the colonies also varied. L-broth agar plates containing antibiotics did not support the growth of any colonies.

57 representative colonies of different sizes were picked up from different plates and streaked on plates containing MG19 agar, L-broth agar containing ampicillin as well as plates with L-broth agar containing tetracycline to confirm the phenotype of the transformants. All the colonies were found to be capable of growing on minimal agar medium as well as on

Table 5. Transformation of *E.coli* W4100 strains by recombinant plasmids from pPK and pBK strains of *E.coli* HB101.

Batch of /Source plasmid pool		No. of transformants* (per ml cell suspension)	Transformation frequency (% of the total cells)
I	pPK	nil	
II		nil	
III		nil	
IV	pBK	7.00×10^2	2.98×10^{-6}
V		2.93×10^2	1.25×10^{-6}
VI		4.95×10^2	2.11×10^{-6}
VII		5.63×10^2	2.39×10^{-6}

*The total number of competent cells in the suspension were 2.35×10^{10} ml^{-1} as determined by growing on arginine supplemented minimal medium.

ampicillin containing medium but not on tetracylcine containing medium as expected, confirming the phenotypes of the transformants as $\underline{Amp^R}$, $\underline{Tet^S}$ and $\underline{Arg^+}$.

These transformant strains carrying recombinant pAT153 in which fragments of pCR1 carrying argF gene are inserted at BamH1 site are named as pBMK strains ($pBMK_1$ - $pBMK_{57}$).

(vi) Isolation of the recombinant plasmid pAT153 carrying argF/G genes

Attempts were made to isolate plasmids from the above strains grown on L-broth containing $35\mu g.ml^{-1}$ ampicillin. The cultures grew in this medium though not very well. However, it was not possible to extract plasmid DNA either by Holmes and Quigley's (1981) method or by Wheatcroft and Williams (1981) method.

Chapter 4 - DISCUSSION

The R prime plasmid, pCR1 is a derivative of pM061 carrying argFG genes but not amiER and is about 135 Kb in size (Rice, 1984). This plasmid was found to be highly stable in *P.putida* PPN 1092 host even after repeated subculturing. Similar observation was also made by Rice (1984). Other R prime plasmids carrying biosynthetic genes argFG were also reported to be highly stable whereas those carrying genes such as trpAB, and amiER were either less stable or unstable (Hedges and Jacob, 1977). As the plasmid was stable in *P.putida* PPN 1092 it was easy to grow the culture in sufficient quantities for large scale isolation of the plasmid. Rice (1984) after trying various plasmid isolation methods used a modified Wheatcroft and Williams' (1981) method for isolation of pCR1 plasmid. Hence, in this study the same method as modified by Rice (1984) was used for making large scale as well as rapid preparations of pCR1 from *P.putida* PPN 1092. However, the purity of the plasmid isolate varied considerably from experiment to experiment. This may be because the method involves two principles such as alkaline denaturation and shearing of chromosomal DNA. The shearing step which was done by vigorous vortex mixing seemed to be adelicate process though the presence of antifoam agent considerably helped in preventing the supercoiled plasmid DNA from being degraded. However, after a few trials one can get used to the required shearing force to get only the RNA and chromosomal DNAs sheared and denatured leaving the plasmid DNA intact. Fairly good yields of reasonably pure plasmid DNA fractions (though the sucrose fractions were rather dilute) were obtained (Fig 5a and 6).

As the plasmid fractions were dilute attempts were made to concentrate them by precipitating the DNA by sodium acetate-ethanol precipitation method. But, surprisingly, the DNA obtained after precipitation and resuspension in TE buffer did not compare either with the plasmid DNA or with chromosomal DNA that was present in the fractions contaminated with chromosomal DNA (Fig. 6). Similar results were also obtained by others (Rice, 1984). This may be due to breaking down of the large DNA chain into smaller fragments. But the fragmentation

seemed to be occurring at the same sites as they showed consistancy in the size of the fragments.

The concentrated plasmid DNA on digestion with BamHl showed only 4 fragments of sizes ranging from approximately 23 to 5Kb in the gel whereas the samples digested with the same enzyme prior to concentration showed large numbers of fragments of varying sizes ranging from approximately 20Kb down to less than 2Kb (Fig. 6). Digestion of intact plasmids with other restriction enzymes such as Pstl (Fig. 7a), Hind III (Fig. 7b) also showed large numbers of fragments.

Fairly good ligation was attained between vector DNAs, pBR322/pAT153 and the restricted fragments of pCRl DNA (Fig. 10 and Fig. 11). pAT153 and pCRl DNA restricted with Pstl showed a broad band in the gel after ligation which measured approximately 3.9-4.5Kb, whereas the same DNAs restricted with BamHl and ligated showed a narrow band measuring about 3.8Kb.

Attempts were made to transform the competent cells of *E.coli* W4100 Arg^- which is an Arg^- strain lacking the arginine biosynthetic gene argF and incapable of growing on minimal medium deficient in arginine. But both pAT153 and pBR322 plasmids (whole plasmids as well as those carrying fragments of pCRl DNA) failed to transform the cells. It may be because of the presence of a strong endonuclease system within the cell. This strain is known to possess restriction enzymes (R.E. Drew, pers. commun.). The foreign DNA that enters the cells may be getting degraded by these enzymes. Hence, an indirect method of introducing the recombinant DNA into this Arg^- strain.

The strategy adopted was transforming another *E.coli* strain, HB101, - an easily transformable strain, selecting recombinant colonies, extracting the recombinant plasmids from these colonies and using them for transforming the strain *E.coli* W4100 Arg.

Transformation of HB101 was quite successful. pAT153 carrying pCRl fragments at Pstl site and BamHl site showed a transformation of frequency of 7.56×10^{-6} and 3.24×10^{-6}, respectively on the basis of total number of cells treated or approximately 2.8×10^3 and 1.2×10^3 transformants per µg DNA, respectively. The transformation frequencies in this study are better than that

obtained by Modi (1984). In a similar study he transformed *E. coli* JA221 with pAT153 carrying fragments of pCR1 DNA and obtained a transformation frequency of 2.9×10^{-6}.

pBR322 which was also supposed to be carrying pCR1 fragments at PstI site failed to transform the cells. This may be due to improper ligation which is evident in Fig. 10. But whole plasmids, both pBR322 and pAT132, showed good transformation. The slightly lower transformation frequency may be due to excess cell concentration (3.7×10^{9} cells ml^{-1}) in the transformation mixture, the ideal concentration being 5×10^{7} cells. ml^{-1} (Maniatis et al., 1982). The transformation by pAT153 carrying foreign DNA at PstI site was more than twice as much as that by pAT153 carrying foreign DNA at BamHI site. This may be because of the differential proportion in which the vector DNA and the insert DNA were mixed.

The percentage of recombinants obtained in the case of pAT153 with insert at PstI site and BamHI site were about 35 and 31, respectively. These rather low values for dephosphorylated vector DNA to which foreign DNA were inserted in which cases more than 90% recombinants were expected. These low values may be due to incomplete dephosphorylation of the vector DNA.

Good yields of the recombinant plasmid DNA were obtained from the recombinant strains (Fig. 12). These plasmid DNAs were pooled and used for transforming the Arg^{-} strain of *E. coli* (W4100). The plasmids with inserts at PstI site (i.e. those from recombinant strains showing tetracylcine resistance and ampicillin sensitivity) failed to transform the competent cells of this strain whereas the plasmids carrying pCR1 fragments at BamHI site (i.e. Amp^{r}, Tet^{S}) showed fairly good transformation (Table 5). The different pools of the latter plasmid preparations showed different transformation frequencies.

An interesting thing observed in these transformant colonies was that they were very slow growing. Though tiny colonies were visible on the minimal agar plates deficient in arginine they took 3 days to grow to the size of the control colonies on arginine containing minimal agar plates. Another interesting observation was that no colony appeared either on ampicillin containing a

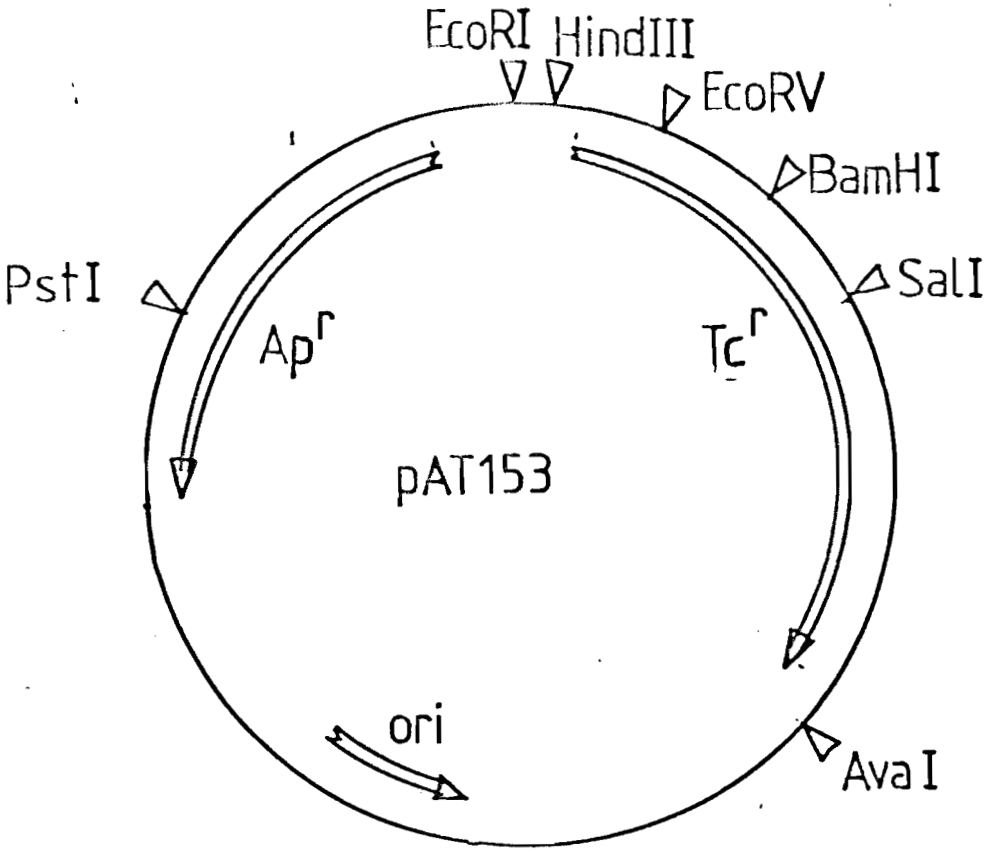
tetracylcine containing L-broth-agar plates. The poor growth on minimal medium and failure to grow on antibiotic containing media might have been due to poor expression of the genes on the plasmid i.e. argFG and ampR. However, when the same colonies were picked and streaked on minimal medium and L-broth-agar containing ampicillin (35µg ml^{-1}) they grew fairly well, though not to the extent of control colonies. Clarke and Laverack (1983) have also obtained similar results when they transformed Arg^- strain, *E.coli* 4100 ArgF with argF carrying R prime plasmids, pAR1 or pM0778. They observed slow growth and also found that the amount of ornithine carbamoyltransferase (OTCase, the product of argF gene) was 2 to 4% of that produced in the parental *P. aeruginosa* strain. However, when they introduced the same plasmids into *P. putida* and *P. aeruginosa* strains the expression of the gene was as good as it was in the parent strain. Their study and that of Mergeay et al. (1978) suggested that the expression of *P. aeruginosa* genes argF and trpAB in *E. coli* was at a reduced level. In contrast to this Mergeay et al. (1978) found that the argE and argH products from *E.coli* genes were expressed in *Pseudomonas fluorescens* at values close to those for wild-type *E.coli*.

Attempts to extract plasmids from the recombinant strains capable of growing on arginine deficient medium and possessing ampicillin resistance and tetracylcline sensitivity were not successful, though the culture grew well in ampicillin containing L-broth. Probably, repeated subculturing on minimal media containing ampicillin as a plasmid amplifying force may yield colonies with stabilised plasmid DNA.

After thus 'rescuing' the strains it would be possible to extract the recombinant plasmids. The size of the inserts could be determined by restricting plasmid(s) with BamHl, at the site of which the pCRl fragment(s) carrying argF/G genes were inserted. From the different fragments, probably, of varying lengths the smallest ones could be selected. Attempts could be made to locate the argF and / argG genes by subcloning and transposon mutagenesis.

If it proves possible to locate the ArgF and / ArgG genes, a start could be made to sequence DNA fragments with the view to identifying the promotor/

operator sequence to compare with the sequence of corresponding *E.coli* genes. R.E. Drew (pers. commun.) has done similar studies with the ami genes in *Pseudomonas*.



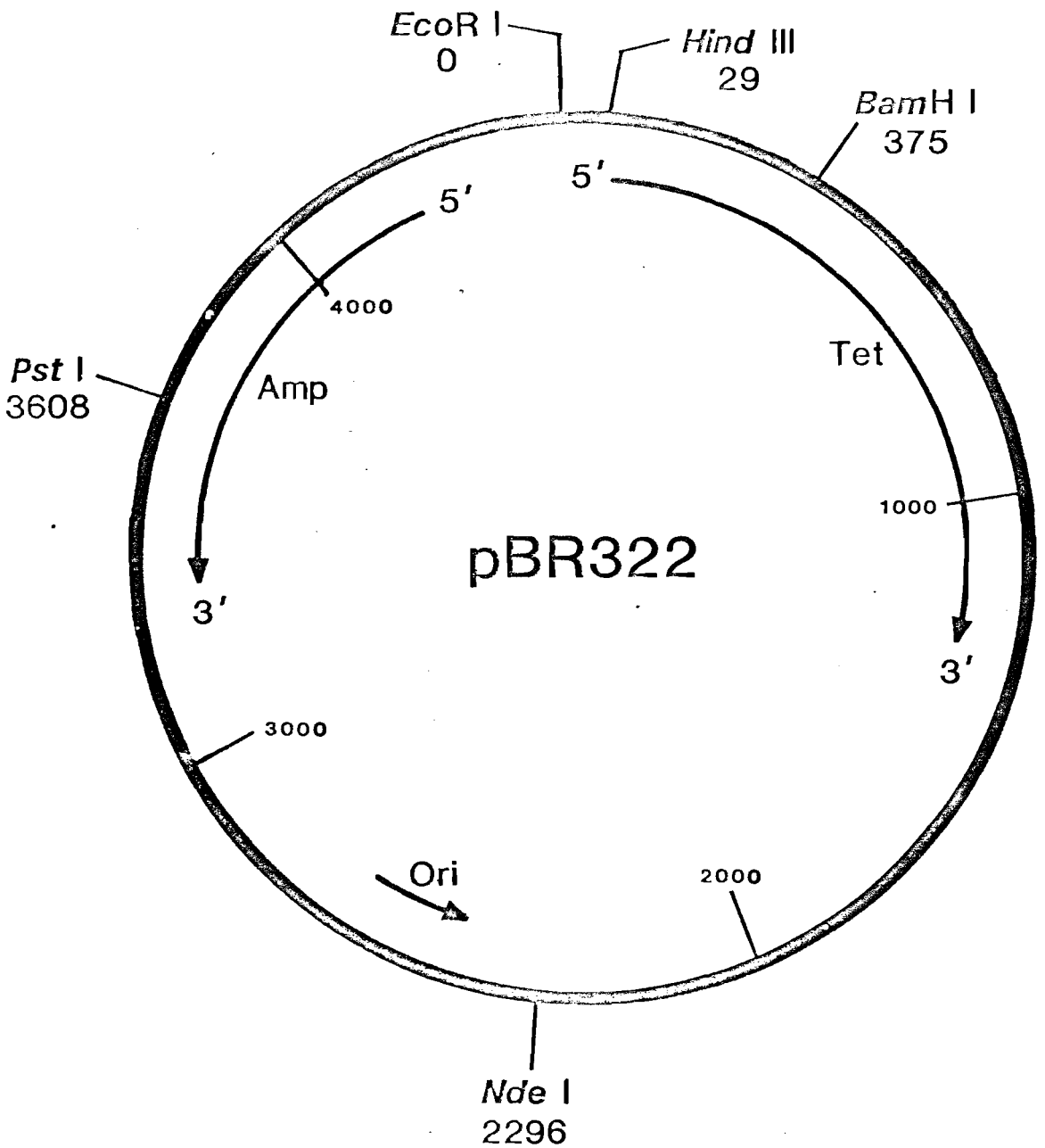
SIZE : 3657 bp

SELECTIVE MARKERS : Tc^r Ap^r

RESTRICTION SITES :

Hind III	29
EcoRV	185
Bam HI	375
Sal I	651
Ava I	1425
Pst I	2904
Eco RI	3657

Restriction Map of pBR322 DNA



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