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A technique for the isolation of Plasmid Deoxyribonucleic acid from Pseudomonas Aeruginosa

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A TECHNIQUE FOR THE ISOLATION OF PLASMID DEOXYRIBONUCLEIC ACID FROM *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

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A Technique for the Isolation of Plasmid Deoxyribonucleic Acid from Pseudomonas aeruginosa

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The Cleared Lysate on Sucrose (CLOS) gradient has been used in these studies as a primary tool in the isolation of plasmid deoxyribonucleic acid (DNA), both sex factor and resistance factor, from Pseudomonas aeruginosa.

A concentration of 1.0 M NaCl in the gradient was shown to keep the chromosome in the "folded configuration". A concentration of 0.05 M NaCl in the gradient was shown to promote chromosomal unfolding. Ten micrograms of ribonuclease (RNase), layered on the gradient, did not affect chromosomal unfolding significantly. Plasmid peak areas from CLOS gradients, presumed to contain the sex factor or resistance factor, were pooled and subjected to Cesium Chloride-Ethidium Bromide gradient analysis. The analysis indicated that only a small percentage of the molecules band in the covalently closed circular (CCC) region of the gradient. The majority of the molecules band in the open circular (OC) and linear regions of the gradient. Contour

length measurements of the linear molecules of the FP2 sex factor indicate that their molecular weight is close to the molecular weight of the plasmid, as reported in the literature.

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CHAPTER I

INTRODUCTION

Pseudomonas aeruginosa was generally regarded as a harmless saprophyte, or at the most, as a microorganism of slight pathogenic importance. Subsequently, it became clear that this bacterium is associated with a variety of infections in man. According to Burrows (1973), the organism may be found in pure culture in abscesses in different parts of the human body. Burrows also reports that cases of pneumonia and endocarditis occur in which Ps. aeruginosa is apparently the only organism responsible. These cases occur rarely. This bacterium is also a contaminant of burn infections. There have been reported cases of gentamicin resistant isolates from burn units in Capetown, South Africa and Atlanta, Georgia (Jacoby, 1975).

Pseudomonas aeruginosa is found in those patients who have cystic fibrosis (CF). The abnormally viscous glandular secretions of these patients block the bronchial pathways. In addition to these secretions, the organism produces a slime which aides in the obstruction of the bronchial pathways. As a result, CF patients usually die of suffocation.

Pseudomonas infections are difficult to treat because of the resistance of the organism to antibiotics. The resistance may be largely due to bacterial plasmid deoxyribonucleic acid (DNA) (extrachromosomal elements). Therefore, plasmids may contribute to this bacterium's success as a pathogen. The above information prompted

our study of the plasmids, both sex factor and resistance factor (R factor), of Ps. aeruginosa.

The technique used most often by other investigators to isolate plasmid DNA, is the Cesium Chloride-Ethidium Bromide (CsCl-EtBr) gradient. However, we directed our efforts toward the isolation of plasmids from a neutral sucrose gradient; a quicker, easier, and less expensive method, and one proven effective in the isolation of plasmids from Staphylococcus aureus (Sheehy and Novick, 1975) and Escherichia coli (Sheehy et al., submitted for publication). This sucrose gradient, is now referred to as a "Cleared Lysate on Sucrose" (CLOS) gradient. We have used the CsCl-EtBr gradient and electron microscopy to further characterize the molecules isolated from the CLOS gradient.

CHAPTER II

REVIEW OF LITERATURE

Lederberg and Tatum (1946) were the first to demonstrate the transfer of genetic material from one bacterium to another by conjugation. The organism used was E. coli. These investigators' experiments launched an era in which E. coli became the most widely used organism for experiments on genetic recombination. Hayes (1952) reported that the capacity to be a genetic donor was determined by a nonchromosomal genetic element which was itself transferred during conjugation. This element was named by its discoverers "F" (for fertility), or the "sex factor" of E. coli K12.

Some years after the discovery of the sex factor, the R factor was discovered. The phenomenon of transmissable drug resistance was discovered in Japan (Watanabe, 1963). During an outbreak of bacterial dysentery in 1955, a strain of the dysentery organism, Shigella dysenteriae, was isolated. The strain was resistant to four antibiotics: Chloramphenicol, Tetracycline, Streptomycin, and Sulfanilamide. Multiply resistant Shigella strains were subsequently found with increasing frequency in clinical practices in Japan. Akiba et al. (1960) suggested that the multiple resistance might be transferred to Shigella from strains of E. coli already resistant. The investigators showed that multiple resistance of this kind was transmitted from one bacterium to another by cell contact. They also reported that the genetic determinants for drug resistance were

transferred *en bloc* by conjugation. The resistance genes, then, were linked as parts of transmissable plasmids, the R factors.

The R factors, reported outside of Japan, were found by Datta (1962) in strains of *Salmonella typhimurium*. These resistant strains were the cause of an outbreak of gastroenteritis in London in 1962. Anderson (1965) also studied the origin and mechanism of transferable drug resistance in the Enterobacteriaceae. Meynell *et al*. (1968), reviewing the work of many investigators, reported that transmissable drug resistance was prevalent in strains of *Salmonella*, *Shigella*, and *E. coli*. This resistance, they reported, was transferred widely among the Enterobacteriaceae, *Vibrio cholera*, *Pasteurella pestis* and *Serratia marcescens*.

Although the study of sex factors and R factors originated with members of the Enterobacteriaceae, many other organisms have been studied also. Genetic and physical tests have allowed other investigators to identify the plasmids of *Ps. aeruginosa*. These tests have shown that this organism harbors a variety of plasmids; sex factors, degradative plasmids, cryptic plasmids and R factors. Holloway (1955) demonstrated conjugation in *Ps. aeruginosa*. After many mating experiments, he reported that conjugation depended on one of the parents of the mating pair having FP2, the sex factor (Holloway, 1969). The sex factor functioned to promote chromosomal gene transfer from one cell to the other. This transfer was detected by the formation of recombinants. Later, Pemberton and Holloway (1973) identified another sex factor, FP3. Matsumoto and Tazaki (1973) discovered

the sex factor, FP5, in another strain of Ps. aeruginosa. The three sex factors differed in the time of transfer of chromosomal markers and their resistance to mercury.

Using density gradient centrifugation, Pemberton and Clark (1973), isolated and characterized the sex factors FP2 and FP3. Separation of plasmid DNA from chromosomal DNA was only one of the problems they encountered because the separate plasmid and chromosomal peaks had to be detected. To detect the peaks, the amount of radioactive label incorporated in the DNA was measured. Tritiated thymidine, the standard DNA label, could not be used because of the difficulty of isolation and maintenance of thymidine requiring mutants. It was also difficult to isolate those strains which did not require thymidine, but still incorporated it. Alternatively, these investigators resorted to a method of labeling both DNA and RNA with tritiated adenine followed by digestion of the RNA with ribonuclease and NaOH. Using this technique and electron microscopy, Pemberton and Clark (1973) characterized FP2 and FP3 by determining density, contour length, and molecular weight of these molecules.

During the physical isolation of these sex factors, other plasmids, which were also covalently closed circular (CCC) molecules, were identified for which no gene functions are known. These molecules were termed "cryptic plasmids". At least three different circular DNA molecules of unknown functions have been isolated from Ps. aeruginosa strain PAO (Pemberton and Clark, 1973).

In addition to cryptic plasmids, there are degradative plasmids harbored by the Pseudomonads. The degradative plasmids occur naturally and specify sets of genes involved in the biodegradation of organic compounds (Gunsalus et al., 1975). Wheelis (1975) indicated five of these organic compounds: Camphor, octane, m-toluate, naphthalene and salicylate. Evidence for the existence of the degradative plasmids has been shown through genetic tests.

The search for R factors in strains of Ps. aeruginosa has also made use of conjugation systems. Bryan et al. (1972) reported that matings with other organisms, e.g., E. coli with Ps. aeruginosa, could permit the detection of R factors which would otherwise be unrecognized. Iyobe et al. (1974) examined strains of Ps. aeruginosa from clinical stocks. They used these strains as donors of drug resistance to detect the presence of R factors. In addition to conjugation systems, physical techniques have allowed investigators to isolate and characterize the R factors. Saunders and Grinsted (1972) used density gradient centrifugation to isolate and characterize RP4, and R factor for Ps. aeruginosa. These investigators characterized the molecule according to its multiple drug resistance, molecular weight and density.

Other investigators used density gradient centrifugation and electron microscopy to study R factors. Bryan et al. (1973) characterized R931. Palchaudhuri and Chakrabarty (1976) characterized RP1. Each investigator used contour length measurements to estimate the molecular weight of these R factors.

Many R factors have been studied, but the techniques for study are similar. Application of these techniques has allowed for a closer study of R factors. The number of drugs for which resistance may be mediated by R factors has grown to eight or more antibiotics, several heavy metals and sulfonamides. Different R factors have different combinations of resistance genes. The mechanism of resistance conferred by these genes varies. Often, an enzyme is produced which destroys the drug or inactivates it by modification, or there is a decrease in permeability to the drug. The emergence of these R factors, which are active against a large number of antibiotics, causes a great deal of concern to clinicians and arouses the interest of many investigators. Thus, a study of the biology of the plasmids of Ps. aeruginosa would be beneficial to our understanding of the regulation, evolution, and function of various genetic elements.

CHAPTER III

MATERIALS AND METHODS

Materials

Bacterial strain. The bacterial strains used in this study are listed in Table 1. The sex factor, FP2, has a molecular weight of 59×10^6 (Pemberton and Clark, 1973). The R factor, RP1, has a molecular weight of 40×10^6 (Palchaudhuri and Chakrabarty, 1976); RP4 has a molecular weight of 62×10^6 (Saunders and Grinsted, 1972). The R^+ strain containing RP4 is resistant to both kanamycin (Km) and tetracyline (Tc).

Media. The synthetic medium used was a minimal salts solution (Curtiss, 1965) supplemented with Casamino Acids (20%), glucose (40%), and adenine (10µg/ml). Antibiotics (20µg/ml of Tc and 40µg/ml of Km) were added to the synthetic medium to select for those cells which were resistant to the specified antibiotics.

Methods of Procedure

Cells were innoculated into the synthetic medium and grown overnight in static culture. The next morning, the cells were diluted 1:10 with the synthetic medium and shaken at 37 C in a water bath. The cultures were allowed to grow for approximately an hour, then they were labeled with 50µCi/ml of ^{3}H-adenine. When an optical density of 0.2 to 0.4 nm (2×10^8 to 4×10^8 cells/ml) was reached (A_{620}), the cells were centrifuged (5 C) at 10,000 rpm for 10 min and resuspended in 1.0 ml

Table 1. Bacterial Strains

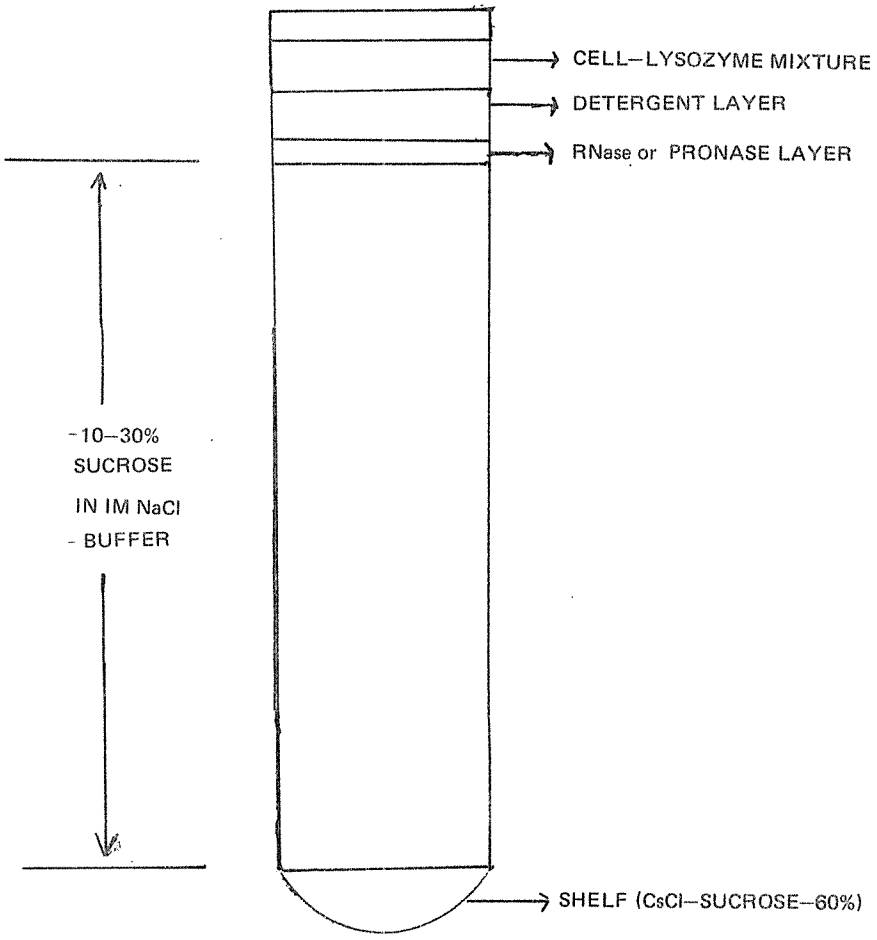
Strain	Plasmid	R-plasmid Genotype	Chromosome Genotype	Source of Plasmid
RS 71	FP2		ad^-	From A. Clark as JC 9005
RS 83	RP1	Tc Km Nm Cb	ad^-	From A. Chakrabarty as AC 35
RS 88	RP4	Cb Km Tc		From G. Jacoby as PU 21 RP4

Abbreviations for R-plasmid drug resistance include: Tc, tetracycline; Km, kanamycin; Cb, carbenicillin and Nm, neomycin.

Strains RS 71 and RS 88 are Ps. aeruginosa strains; RS 83 is a Ps. putida strain.

of cold 5% sucrose resuspension buffer, pH 8.0. This buffer contained 0.2 M KCN, 1.0 M NaCl, 0.01 M ethylenediaminetetraacetic acid (EDTA) and 0.01 M tris (hydroxymethyl) aminomethane (Tris-HCl). A 0.4 ml portion of the concentrated cell suspension was mixed with 0.1 ml (10mg/ml stock) of lysozyme prepared in 0.01 M Tris-HCl and 1.0 M NaCl, pH 8.0, to give a final volume of 0.5 ml. A 0.1 ml sample of this mixture was immediately layered onto a 10.5 ml or 4.4 ml 10-30% neutral sucrose gradient maintained at 5 C. The final components of the sucrose gradient were a 0.5 ml or 0.2 ml 60% sucrose-CsCl shelf, 10.5 or 4.4 ml of 10-30% sucrose, 0.2 ml of detergent, and 0.1 ml of the cell and lysozyme mixture (Fig. 1). For the addition of ribonuclease (RNase) on the gradient, a stock solution was made consisting of 1mg/ml or 5mg/ml of RNase buffered in 7% sucrose, 1.0 M NaCl, 0.01 M Tris-HCl, and 0.01 M EDTA at pH 8.0. This stock solution was properly diluted to give the desired concentration. A 0.1 ml aliquot of the RNase solution was layered on the gradient before the addition of the detergent. The detergent used was Triton X-100 (4% Triton in 5% sucrose). Both the detergent and the gradient contained 1.0 M NaCl, 0.01 M Tris-HCl and 0.01 M EDTA. To test the effects of other molarities of NaCl on the chromosomal profile, concentrations of 0.5 M or 0.05 M NaCl were added in the gradients. After layering, the gradients were allowed to stand for 10 min at 5 C. They were spun at 5 C in Beckman SW 50.1 (5 ml capacity) or Beckman SW 41 (11 ml capacity) swinging bucket rotor in Beckman L5-50 or L5-65 ultracentrifuges. Gradients were collected from the top with an Auto Densi-Flow II connected to a polystaltic pump. After centrifugation, 15 drops per

Fig. 1. A profile representing the components of the CLOS gradient.


CELL–LYSOZYME MIXTURE
DETERGENT LAYER
RNase or PRONASE LAYER
-10–30%
SUCROSE
IN IM NaCl
- BUFFER
SHELF (CsCl–SUCROSE–60%)

fraction were collected from 11 ml gradients and 10 drops were collected per fraction from 5 ml gradients. The fractions were collected into plastic trays. A 0.025 ml sample was taken from each fraction in the tray and placed into a tray containing 0.025 ml of 1.0 M NaOH made in 7% sucrose. The NaOH treated samples were incubated for 3 hr at 37 C to hydrolyze the RNA. The remaining untreated sample was refrigerated. After incubation, the samples were spotted onto paper strips. Strips were air dried, washed for 10 min each in 5% trichloroacetic acid (TCA), 10% TCA, 95% ethanol (ETOH), acetone, and air dried. The strips were cut, placed into vials containing toluene-PPO, and the samples were counted in a Beckman scintillation counter.

CsCl-EtBr Gradient Analysis. After analyzing the profiles obtained from the sucrose gradient, those regions which represented peak plasmid areas were pooled. Pooled samples came from the refrigerated trays. The pooled samples were diluted with 0.1 M phosphate buffer. A precalculated amount of sample and CsCl were mixed, and 0.1 ml of EtBr (10mg/ml) was added to give a final volume of 6 ml to the gradient. Gradients were spun for 36 hr at 20 C in Beckman SW 50 Ti fixed angle rotor in Beckman L5-50 or L5-65 ultracentrifuges. These gradients were collected from the bottom into trays containing 0.050 ml samples of 1.0 M NaOH in 7% sucrose. The samples were incubated at 37 C for 3 hr to hydrolyze the RNA. After incubation, 0.025 ml samples were spotted onto paper strips, washed, and counted as previously noted. After analysis of these profiles, those areas