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I am submitting herewith a thesis written by Margaret Carlisle Chappell, entitled "Development of a Technique for the Isolation of Plasmids from Food-Related Pseudomonas fluorescens." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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DEVELOPMENT OF A TECHNIQUE FOR THE ISOLATION OF PLASMIDS FROM FOOD-RELATED *PSEUDOMONAS FLUORESCENS*

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

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December 1987

AG-VET-MED.
Thesis
87
.C464

DEDICATION

To my father and mother, without whose continued support and encouragement this work might never have been pursued.

ACKNOWLEDGEMENTS

My sincere appreciation is extended to Dr. P. M. Davidson for his support and guidance throughout the course of this study. I would also like to thank Drs. G. L. Christen and G. A. Baumbach for their advice and encouragement as committee members.

Special thanks go to my office mates Eric Line, Laure Larkin, Emi Rico-Munoz and Clyde Hoskins for their friendship and understanding and to fellow students and staff Mohammed Amin, Nancy Bartley, Becky Guthrey and Lynda Gayle Hasty for their help and support.

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ABSTRACT

Four strains of Pseudomonas fluorescens were prepared for gel electrophoresis by several methods to detect plasmids. The procedure of Anderson and McKay (1981) produced DNA preparations most amenable to gel electrophoresis when compared to the plasmid reference strains E. coli V517 and P. putida NAH+. No plasmids were detected in the range of 1.4-35.8 megadaltons in any of the strains of P. fluorescens. Attempts to transform the protease negative strain, RM14, with DNA from the protease positive strains proved to be successful only when the recipient cells were exposed to 1,000 times the reported maximum level of DNA that can be taken up by competent cells. However, plasmid profiles of these transformants showed no evidence of the uptake of a plasmid.

CHAPTER I

INTRODUCTION

Pseudomonas fluorescens is a common spoilage agent of many refrigerated proteinaceous foods due to its ability to grow, even at 0° C. Development of slime and off-odor is characteristic of spoilage associated with the microorganism and can be attributed to the extracellular enzymes it produces. Some of these proteolytic enzymes are extremely heat resistant, surviving both UHT and HTST pasteurization (Law et al., 1977; McKellar, 1981).

Knowledge concerning the genetic regulation of enzyme production by *P. fluorescens* is limited. *Pseudomonas aeruginosa* and *P. putida*, which have been shown to be phylogenetically related to *P. fluorescens*, have been characterized to a greater extent (Palleroni et al., 1973, Clarke and Richmond, 1975). For example, the location of various genes on the chromosome of *P. aeruginosa* has been well documented (Holloway et al., 1971, 1979). Therefore, the existing knowledge concerning the genetics of *P. aeruginosa* may be applied to the techniques available for studying the genetics of *P. fluorescens*.

The presence or absence of extrachromosomal DNA elements, known as plasmids, in *P. fluorescens* has a direct impact on the type of recombinant DNA techniques that are applicable to the organism. The presence of a plasmid which codes for this extracellular heat resistant protease would enable scientists to construct a new tool, called a DNA probe, to be used in detecting and enumerating specific bacteria.

The objective of this project is to establish a procedure for the isolation and characterization of plasmids from food-related *P. fluorescens* strains.

CHAPTER II

REVIEW OF THE LITERATURE

1. The Genus *Pseudomonas*

Classification and Significance

The genus *Pseudomonas* is composed of gram negative microorganisms, which are characteristically aerobic, motile and rod-shaped. Pseudomonads are found commonly in soil and water and can utilize a wide variety of organic compounds to produce energy (Clarke and Richmond, 1975). All species create energy via respiration with oxygen as the terminal electron acceptor. Pseudomonads are able to utilize simple nitrogenous compounds and may synthesize their own growth factors and vitamins. Therefore complex microbiological media are not required for maintenance.

Species of *Pseudomonas* that are of the greatest interest to scientists are *P. aeruginosa*, *P. putida* and *P. fluorescens*. *Pseudomonas aeruginosa* is probably the most widely studied species of the family *Pseudomonadaceae*. It is an opportunistic pathogen and may establish infections in patients whose natural resistance has been compromised. Burn patients and those on immunosuppressive drugs are at the greatest risk (Stanier et al., 1976). *Pseudomonas putida* is most noted for its abilitiy to metabolize various organic chemicals such as toluene, naphthalene and salicylate (Sayler et al., 1985; Johnson and Gunsalus, 1977). Researchers are extremely interested in the possible environmental applications of this species, specifically, those in which the microorganism is used to degrade industrial pollutants in wastewater.

Pseudomonas fluorescens, so named for the greenish fluorescent pigment it produces, is psychrotrophic and grows well even at 0° C. Because of its ability to grow at low temperatures, this species is a common contaminant of refrigerated foods. Spoilage associated with the organism is caused primarily by the proteolytic and lipolytic enzymes it produces. Evidence of spoilage by P. fluorescens includes slime and an off-odor in meats (Ayres et al., 1950) and coagulation and off-flavors in dairy products (Fairbairn and Law, 1986a). The ability of P. fluorescens to grow and compete well against a variety of other microflora makes it an important spoilage agent in proteinaceous foods.

Examples of Spoilage

In studies as early as 1950, the characteristic development of slime and off-odor on poultry described as "sour" and "tainted" had been attributed to several species of Pseudomonas (Ayres et al., 1950). A review article by Ayres in 1960 addresses the spoilage of poultry, ground beef, sausage, fish and eggs by organisms of the genus Pseudomonas (Ayres, 1960).

Dainty et al. (1975) researched the changes in the protein content of beef spoiled by Pseudomonas. In repeated experiments, this genus continually produced a slime of beef slices and an off-odor. They found that proteolysis by the organism most affected the sarcoplasmic and myofibrillar protein fractions of the muscle. Jay and Shelef (1976) stated "that Pseudomonas spp. constitute the single most predominant and important group of bacteria in the refrigerator spoilage of fresh meat and poultry." They used 20 different strains of Pseudomonas

isolated from spoiled beef to study the breakdown of beef actomyosin at 5° C. Some species of Pseudomonas were shown to be capable of destroying as much as 82% of the protein.

Liston (1960) reported the distribution of different types of bacteria on fish caught in three areas of the Pacific Ocean. In all areas Pseudomonas was isolated in greater numbers than any other genera.

The effect of the extracellular enzymes produced by these psychrotrophic bacteria on the acceptance and keeping quality of dairy products is of considerable interest to the dairy industry. Some specific effects on flavor and shelf life are addressed in detail later in this chapter.

One explanation for the predominance of pseudomonads in the spoilage of meat and dairy products is that much of the natural flora present in the products are mesophiles which grow poorly or not at all at refrigeration temperatures. Pseudomonas is a strict aerobe and produces all of its own growth factors. The high humidity and high solubility of gases, namely oxygen, in the refrigeration unit provide an ideal environment for the surface growth of these psychrotrophs (Jay and Shelef, 1976). Gill and Newton (1977) reported that pseudomonads have a greater affinity for oxygen than the other microflora present on meat enabling them to grow faster than competing species. Growth of Lactobacillus, Streptococcus and Escherichia is not favored in this highly oxygenated, low temperature environment. Therefore, spoilage of refrigerated meats and poultry by psychrotrophic Pseudomonas is of considerable importance. To curtain the potential loss of food

products, it is necessary to understand this organism's metabolism and production of enzymes at refrigeration temperatures.

2. Protease of P. fluorescens

Although non-sporeforming microorganisms are readily killed by pasteurization, some of their enzymes may not be totally inactivated. The proteolytic enzymes of P. fluorescens for example, have been found to survive certain heat treatments given to milk. Resultant spoilage of dairy products may be due to the breakdown of milk proteins by these proteases.

Heat Resistance

The proteases produced by P. fluorescens are extracellular and often extremely heat resistant. Because of this heat resistance, these enzymes are a particular problem in dairy products. At present there are several types of heat treatments approved by the FDA for pasteurization of milk. The two most commonly used are LTLT, low-temperature long-time pasteurization where raw milk is held for 30 min at 145° F (62.8° C), and HTST, high-temperature short-time pasteurization, where milk is heated at 161° F (71.7° C) for 15 sec. A relatively new pasteurization treatment, ultrahigh-temperature or UHT, requires that the milk or milk products be heated at or above 180° F (137.8° C) for at least 2 sec either before or after packaging (21 CFR 131.3, April, 1987; Campbell and Marshall, 1975). Several of the proteases produced by P. fluorescens are able to withstand UHT and HTST pasteurization. Law et al. (1977) reported that P. fluorescens AR11

produced a protease which was resistant to UHT treatment and which subsequently caused gelation of the UHT milk. Further research with this organism, renamed P. fluorescens NCDO 2085, showed that the protease had a D value (time to reduce activity by 90%) of 1.6 min at 74° C and 1.0 min at 140° C. Furthermore, this protease retained 70% of its original activity after a standard UHT treatment (Fairbairn and Law, 1986b).

Other researchers have found similar results with proteases from different strains of P. fluorescens and from different organisms. Mitchell et al. (1986) characterized eight extracellular proteases produced by psychrotrophs isolated from raw milk. Six of these strains were identified as P. fluorescens and two as Serratia marcescens. Seven of the proteases retained partial activity after conventional pasteurization and also were able to withstand UHT treatment. Their D values at 140° C ranged from 2-300 sec.

An investigation by Adams et al. (1975) compared the heat resistance of the protease from P. fluorescens MC60 to the heat resistance of spores of Bacillus stearothermophilus and putrefactive anaerobe (PA) 3679. They found that the protease had a D value of 90 sec at 149° C which was approximately 4,000 times more heat resistant than spores of B. stearothermophilus and 400 times more heat resistant than spores of PA 3679. By comparison, milk can be sterilized by a heat treatment of 149° C for 4 sec, but less than 10% of the MC60 protease would be inactivated by this treatment. Further studies with this protease were conducted by Barach and Adams (1977). They compared

the thermostability of the MC60 protease to that of thermolysin, a protease produced by B. thermoproteolyticus, and discovered that the compounds had similiar amino acid percentages, heat inactivation parameters and composition.

Patel et al. (1983) compared the heat stabilities of seven extracellular proteases isolated from strains of P. fluorescens found in raw milk. They heated samples at three temperatures and various times and found that, in all instances, the proteases were more susceptible to inactivation at 50° C than at any other temperature. This was termed "low temperature inactivation" or LTI. They proposed that a 50° C the enzyme structure underwent a conformational change which caused an irreversible loss of activity, and that this change does not occur at higher temperatures. At higher temperatures these enzymes are considerably more stable than at 50° C.

Characterization

The majority of these extracellular proteases are metalloproteases with molecular weights usually ranging from 40,000 to 50,000 daltons (Fairbairn and Law, 1986a). However, the protease from P. fluorescens P26 is somewhat smaller, with a mass of 26,000 daltons. Barach and Adams (1977) suggested that heat stability may be favored in the proteases which contain calcium in their native conformation.

The temperature range for optimum enzyme activity is species dependent and ranges from 30-50° C (Fairbairn and Law, 1986a). The

protease from P. fluorescens AR11, however, exhibits as much as 33% of its maximum activity at 4° C and 72% of the maximum activity at 20° C (Alichanidis and Andrews, 1977).

Heat stability also varies with the medium used for the suspension of the enzymes. The heat stabilities of the proteases of P. fluorescens P26, AFT 36, and B52 were all enhanced in milk or milk-based medium while the enzymes were more heat labile in buffer solutions (Mayerhofer et al., 1973; Stepaniak et al., 1982; and Richardson, 1981). It was postulated that the presence of calcium in the medium played a vital role in maintaining the heat stability of the proteases. Enzyme activity was markedly reduced when the chelating agent EDTA (ethylenediaminetetraacetate) was present. However, when metal ions such as Zn^{2+} or Ca^{2+} were added, partial activity of the enzymes was restored (Stepaniak et al., 1982; Richardson, 1981; Patel et al., 1986).

Effect of Nutrients on the Production of Protease

Recent research has indicated that the production of protease by Pseudomonas is not the result of nutrient limitation, as previously thought, but in fact occurs at a maximum in medium supplied with excess carbon, nitrogen and phosphate (McKellar and Cholette, 1984, 1985). Milk proteins, such as casein, were also found to be unnecessary for production of the protease (McKellar and Cholette, 1984; Rowe and Gilmour, 1983). However, calcium is essential for the production of protease by the organism. Calcium stimulated protease production more

than any other single nutrient, although the mechanism for production was not explained (McKellar and Cholette, 1985; Amrute and Corpe, 1978).

Torrie et al. (1983) observed that the protease-deficient mutant of P. fluorescens 32A, named RM14, was unable to grow when sodium caseinate was used as the sole source of carbon. This mutant did compete with the parent strain when both were inoculated into skim milk and grown at 6° C and 20° C. RM14 grew at a slightly slower rate than did the parent at 20° C while at 6° C, the protease producing parent strain showed a 2 to 3 fold greater cell yield. Therefore, the activity of the extracellular protease is necessary for P. fluorescens to grow on milk proteins but not essential for survival. Production of the protease apparently does confer a selective advantage on the organism (Torrie et al., 1983).

Effect on Dairy Products

The heat resistance of the proteases can contribute to a decreased shelf life of dairy products. The product most affected by these proteolytic enzymes is UHT milk. McKellar (1981) concluded that UHT milk was twice as sensitive to proteolysis by these enzymes as HTST pasteurized milk. He suggested that the high temperature exposed new enzyme substrate sites on protein molecules in the milk. McKellar, however, did not take into account the influence of storage temperature on the amount of proteolysis observed in UHT milk. UHT milk is commonly stored at room temperature, which is much closer to the optimum temperature for enzyme activity than HTST milk, which is refrigerated.

McKellar (1981) stated that the intensity of off-flavors in the UHT milk increased in proportion to the amount of proteolysis. Mayerhofer et al. (1973) reported bitterness as the primary off-flavor in skim milk to which protease had been added.

Law et al. (1977) and Richardson and Newstead (1979) investigated the gelation effect on UHT milk of proteases from P. fluorescens AR11, B12 and B52. Gelation of the UHT milk was found to resemble the coagulation by rennin of pasteurized milk used for making cheese. They postulated that the proteases degrade K-casein to para-K-casein, causing gelation of the milk. B-casein was also hydrolyzed, and the bitter peptides which accumulated from the breakdown of this milk protein may account for the off-flavors reported (Fairbairn and Law, 1986a). Law et al. (1977) hypothesized that gelation time was dependent on the number of organisms present before heat treatment. Richardson and Newstead (1979) stated that UHT milk containing more than 1-2 ng/ml B52 protease would be unlikely to have a storage life of greater than three months.

Many researchers (Alichanidis and Andrews, 1977; Fairbairn and Law, 1986a; Patel et al., 1983; Richardson and Newstead, 1979; Stepaniak et al., 1982) stress concern over the current practice of storing raw, cooled, bulk milk for several days before heat treatment. This procedure apparently facilitates the growth of psychrotrophic organisms and the production of extracellular heat resistant enzymes.

White and Marshall (1973) conducted an investigation into the shelf life of several dairy products made from milk which had been inoculated with either P. fluorescens P26 or its protease. The

organoleptic properties of butter, cheddar cheese, cottage cheese, vanilla ice cream mix and homogenized milk were evaluated by an expert three member panel using the Scoring Guide of the American Dairy Science Association or by a semi-trained panel of 12-14 people. Either protease or the organism was added to each product 12 h before manufacture. The milk plus the protease was repasteurized at 63° C for 30 min before tasting. The quality of the milk, butter and ice cream mix were not significantly effected by the action of the protease. However, the flavor quality of the cheddar cheese and the cottage cheese was significantly reduced when the milk used to make either product contained protease or the organism.

3. Genetic Control of Protease Production

Little is known concerning the genetic regulation of enzyme production by *P. fluorescens*. DNA-DNA and DNA-rRNA hybridization studies have shown that there are five sharply defined RNA homology groups of *Pseudomonas* (Palleroni et al., 1973; Clarke and Richmond, 1975). *Pseudomonas fluorescens*, *P. aeruginosa*, *P. putida*, and the fluorescent plant pathogens comprise Group 1. They have an 87-105% DNA homology among species. Therefore, it is possible that the organisms within this group contain such similar DNA sequences that the production of proteolytic enzymes by these bacteris is genetically controlled in the same manner.

Pseudomonas fluorescens has been subdivided into several biotypes dependent on two main biochemical characteristics, the capacity for denitrification and the synthesis of levan from sucrose. This internal subdivision, however, is far from satisfactory since some strains are incapable of both of these characteristics and, in the opinion of Clarke and Richmond (1975), appear to deserve species status.

Chromosomal Control

Pseudomonas aeruginosa is by far the most researched among Pseudomonas species. The genetic organization of this organism has been studied using conjugation, transduction, and transformation as processes of chromosomal transfer. Holloway et al., (1971) presented a partial linkage map for P. aeruginosa PAO. This map was derived from the results of interrupted mating experiments using the transducing phage of P. aeruginosa, F116. Phage F116 is probably the most commonly used temperate phage of P. aeruginosa, since it rarely is affected by DNA restriction enzymes in the recipient strain (Holloway et al., 1971, 1979; Clarke and Richmond, 1975). In 1979, Holloway published a more complete linkage map of the chromosome of P. aeruginosa PAO and a partial map of P. putida. The new map for P. aeruginosa included the location of genes which produced extracellular protease. Holloway explained that the exact location of the genes was known, but that a relationship to flanking markers had been determined by coinheritance. The first marker for extracellular protease was near minute 10 on the new map, and the second marker was near minute 35 and was 93% coinherited with a marker for tryptophan utilization.

A protease-deficient mutant of P. fluorescens which required tryptophan for growth was isolated by Torrie et al. (1983) using ethyl methanesulfonate. The protease-deficient tryptophan revertant of this mutant was named RM14. Torrie and coworkers (1983) speculated that the loss of protease production occurred from a chromosomal deletion and that the tryptophan positive revertants could have arisen from another mutation in a different gene.

Plasmid Control

While evidence exists that protease production is chromosomally directed, it does not preclude the possibility that the production of some types of extracellular proteases may be plasmid linked. Plasmids, or extrachromosomal pieces of DNA which either replicate autonomously or integrate into the chromosome, can confer many different characteristics on their host. Control of conjugation, resistance to antibiotics, production of enterotoxins and utilization of organic compounds for energy are among the most common phenotypic traits encoded on plasmids. Plasmids may be lost spontaneously from the host if selective pressure, such as the presence of an antibiotic, is removed from the environment. Often environmental strains of Pseudomonas contain plasmids that encode for the resistance to metals or the degradation of toxic wastes, characteristics that are vital for the survival of the organism (Trevors, 1985).

Plasmid activity in Pseudomonas varies widely. In 1973, Dunn and Gunsalus discovered a plasmid coding for the enzymes which oxidized

naphthalene in P. putida. Johnston and Gunsalus (1977) studied the plasmids that confer the ability to utilize either naphthalene (NAH^+), salicylate (SAL^+) or toluate (TOL^+) from strains of P. putida by transferring the plasmids to strains without plasmid activity.

P. aeruginosa commonly carries plasmids which confer resistance to drugs, UV light, mercurials and other metals, as well as inhibition of phage growth and inhibition of the fertility of other plasmids (Fennewald et al., 1978; Holloway et al., 1971; Clarke and Richmond, 1975). The plasmids of P. aeruginosa may be divided into two groups, those that are transferable between species of Pseudomonas and those that are transferable to Escherichia coli and other enteric bacteria. They can be divided further into eight groups based on their incompatibility with each other. Incompatibility groups contain plasmids that cannot coexist stably or replicate when placed in a cell together. It is assumed that these plasmids share some common genetic element that allows for the expression of only one phenotype at a time (Bukhari et al., 1977; Clowes, 1972). For example, plasmids RP1 and RP4 may be transferred to a wide range of other species and genera and are termed "promiscuous" plasmids. Since organisms of other genera readily accept these two plasmids, it is assumed that these organisms do not contain plasmids of a similar incompatibility group, or with similar replication systems (Clarke and Richmond, 1975). However, plasmids of the P-2 incompatibility group are not transmissible to E. coli and are considered to have a limited host range.

Fennewald et al. (1978) investigated the Inc P-2 incompatibility group by isolating plasmid fragments through a "laborious" ultracentrifugation technique involving a Hoescht dye. They then attempted unsuccessfully to transfer them into strains of P. aeruginosa and P. putida which contained no plasmids. They attributed the difficulties they encountered to two factors. First, they found that these plasmids were extremely large, greater than 200 megadaltons. Second, electron microscopy demonstrated that all of the plasmid DNA was composed of linear fragments, which is incapable of transforming other bacteria. Further research on the replication functions encoded on Inc P-2 plasmids of P. aeruginosa has been done by Prince and Barlam (1985).

Plasmids that offer the host a selective advantage over other organisms in a harsh environment are seen frequently in Pseudomonas. Some Pseudomonas strains which have been isolated from chemical wastewaters include Pseudomonas species BS831 and BS835, both of which can utilize Σ-caprolactam, an industrial pollutant, as a sole carbon source (Boronin et al., 1984). Pseudomonas fluorescens LB300, which is resistant to chromate, was isolated from sediment in the upper Hudson River (Bopp et al., 1983). These distinctive characteristics are both plasmid directed. The plasmid coded degradation of dibenzothiophene by Pseudomonas is of interest to researchers studying the removal of organic sulfur from oil (Monticello et al., 1985). The genetic manipulation of these degradative pseudomonads also holds great promise for environmental scientists who see these organisms as vehicles which can be used to degrade pollutants from wastewater.

Wretlind et al. (1977) studied the extracellular proteases produced by P. aeruginosa PAKS-1 and postulated that the genetic information for the synthesis of proteases might be located on plasmids. However, they were unsuccessful in their attempt to eliminate protease production by various methods used to cure cells of plasmids. Ultracentrifugation of total bacterial cell DNA using sucrose density gradients failed to show any plasmid DNA in this strain. His results reinforce the previous presumption that extracellular protease production is coded on the chromosome.

4. Techniques for Studying Plasmids

Methods of Detection and Isolation

Many procedures exist for the isolation and characterization of plasmid DNA. Most methods are based on the size difference between plasmid and chromosomal DNA and take advantage of conditions which denature the large chromosome and leave the small plasmid DNA intact (Clowes, 1982; Trevors, 1985).

The first step of any method entails the formation of spheroplasts by the addition of lysozyme and ethylenediamine tetraacetate (EDTA) in buffered sucrose (Clowes, 1972; Maniatis et al., 1982; Trevors, 1985). The addition of sodium dodecyl sulfate (SDS) solubilizes the protein present and forms a complex with chromosomal DNA. Salts such as K^+ and Na^+ precipitate the SDS-DNA complex which is removed by centrifugation. Proteins and the remaining SDS are removed by an extraction with buffered phenol and pelleted by centrifugation.

The aqueous supernatant then can be extracted with chloroform or a chloroform-isoamyl alcohol mixture to remove phenol and help separate the aqueous and organic phases. Then the plasmid DNA is precipitated at -20° C from the aqueous phase by the addition of cold ethanol or isopropanol. Pancreatic ribonuclease A (RNase) is sometimes used to degrade contaminating RNA in the sample. Gel electrophoresis is the most common method employed to separate the plasmid DNA which then is visualized by the dye, ethidium bromide.

There are numerous modifications of these steps. Kado and Liu (1981) used a lysing solution devoid of lysozyme for cellular disruption and destroyed chromosomal DNA by heating the cell lysate to 50-65° C. Birnboim and Doly's procedure (1979) optimized the narrow pH range (12.0-12.5) at which chromosomal DNA is denatured to remove it from the plasmid DNA. Wheatcroft and Williams (1981) developed a rapid method of isolating plasmid DNA by using a buffered antifoam emulsion and alkaline SDS to isolate the plasmid DNA. They then used ultracentrifugation of the DNA on a sucrose gradient as a method of purification and characterization.

One other method frequently used for isolation of plasmid DNA is dye-buoyant density centrifugation (Clowes, 1972; Maniatis et al., 1982; Trevors, 1985). A cesium chloride-ethidium bromide mixture is added to a crude cell lysate containing plasmid and chromosomal DNA and the solution is separated by ultracentrifugation. The ethidium bromide intercalates within the helix of the DNA and fluoresces under an ultraviolet light. Therefore the chromosomal and plasmid DNA can be

visualized as distinct bands in the centrifuge tube by illumination of the sample. Researchers that have employed this technique include Klaenhammer et al. (1978) and Holmes and Quigley (1981). Other methods of plasmid isolation include hydroxylapatite chromatography and adsorption on nitrocellulose (Clowes, 1972; Trevors, 1985).

Elimination from Cells

Removal of the selective pressure for the existence of a certain plasmid may result in the spontaneous loss of that plasmid. Plasmid loss may be increased by subjecting the strain to certain treatments or curing agents.

Some treatments are more effective at curing one type of plasmid than another. Acridine orange is frequently used to eliminate F plasmids, or those that control the process of conjugation. For example, acridine dyes are effective in curing E. coli of its sex factor F plasmid which codes for production of the sex pili (Salisbury et al., 1972).

Acridines, however, have little effect on drug resistance plasmids, termed R factors. Resistance plasmids usually code for an enzyme or protein that modifies either an antibiotic or its site of action thereby rendering the host unaffected by the drug (Maniatis et al., 1982). Salisbury and coworkers (1972) found that SDS was selectively effective at curing some of the R plasmids from strains of E. coli but ineffective on other plasmids and on some whole strains. They postulated that SDS selects for plasmidless clones rather than

producing cured cells. Sonstein and Baldwin's results (1972) agreed. They allowed 38 strains of Staphylococcus aureus, all of which produced the B-lactamase (penicillinase) R plasmid, to grow in a medium supplemented with SDS. Only two of these strains showed a significant increase in the production of plasmid negative strains. The rate of curing of these two strains, however, was near 100%.

Other researchers have reported similiar results using ethidium bromide to eliminate R plasmids from enterobacteria and staphylococci. Bouanchaud et al. (1968) were able to eliminate penicillinase production in mercury resistant staphylococci but not in mercury sensitive ones. In Salmonella derby LA71, only one of the three resistant characters was totally (100%) eliminated by ethidium bromide, while no elimination of resistance was noticed in two other strains, E. coli LA49.R15 and S. paratyphi B LA43.R8 (Bouanchaud et al., 1968). Rubin and Rosenblum (1971) were unable to achieve greater than 58% elimination with ethidium bromide in any of the S. aureus strains used.

Other curing agents include ultraviolet light, crystal violet, rifampicin, and the use of thymine starvation (Clowes, 1972). Johnston and Richmond (1970) studied the effects of rifampicin on the penicillinase plasmids of S. aureus but reported only 20% elimination of the resistance genes after overnight growth with the antibiotic.

Gene Transfer

Gene transfer is frequently accomplished by means of conjugation, transduction, or transformation. Conjugation requires cell to cell

contact. A conjugative gene transfer procedure described by Lee and Richmond (1969) was used by Sykes and Richmond (1970) to transform *E. coli* K12 strains 1822 and 3425 to carbenicillin resistant strains. The procedure involved cell to cell contact between the *P. aeruginosa* host and the *E. coli* recipient.

Transducing phages have been used as vehicles of transfer in numerous experiments (Holloway et al., 1971; Clarke and Richmond, 1975). Both Chakrabarty et al. (1967) and Gunsalus et al. (1968) investigated the mapping of tryptophan genes in *P. putida* using the transducing phage of *P. putida* pf16.

Transformation implies the transfer of genes which code for a specific characteristic, such as the plasmid coded degradation of toluene. The recipient strain, deficient in this characteristic, is treated to facilitate the uptake of plasmid DNA so that the strain becomes capable of degrading toluene. No cell-to-cell contact is necessary and no transducing phage is needed as a vector.

Transformation involves treating the recipient cells with a calcium chloride solution, adding the plasmid DNA of interest to the competent cells, then subjecting the cells to an ice bath followed by a heat pulse (Maniatis et al., 1982; Lederberg and Cohen, 1974; and Cohen et al., 1972). Cells are plated onto a selective medium specific for the transformants. This procedure has produced successfully the transformation of *S. typhimurium* (Lederberg and Cohen, 1974) and *E. coli* (Cohen et al., 1972) from antibiotic susceptible bacteria to strains resistant to certain antibiotics.

Use of Plasmids in Biotechnology

Recent advances in biotechnology have made it possible to develop tests which screen for a specific DNA sequence and its host organism using specially prepared probes. Plasmids are often used to construct these probes which are then used to seek out and hybridize to complementary sequences in an unknown sample (Mulcahy, 1986). Such DNA-DNA hybridization techniques have been used successfully to detect the catabolic genotypes NAH^+ and TOL^+ of P. putida in environmental samples (Sayler et al., 1985), the P. fluorescens species as a whole (Festl et al., 1986) and to identify Salmonella in foods (Fitts, 1985, Fitts et al., 1983).

Sayler and his colleagues (1985) utilized existing DNA hybridization techniques to construct a DNA probe using the P. putida plasmid coding for toluene degradation. The plasmid probe was able to detect the existence of the organism carrying the NAH^+ plasmid and two other P. putida strains carrying derivatives of the toluene degradative pathway.

Festl and coworkers (1986) constructed their plasmid probe for P. fluorescens from rRNA isolated previously from an enzyme digest of P. aeruginosa DNA (Schleifer et al., 1985). Their plasmid, dubbed pHF360, hybridized exclusively to chromosomal DNA from bacteria which are considered part of the P. fluorescens RNA homology group which includes P. aeruginosa, P. putida, and P. stutzeri. It did not cross-react with DNA from other RNA homology groups of Pseudomonas or from other genera.

Salmonella has no known toxin or virulence plasmids that can be used to construct a probe for all Salmonella species. Fitts and coworkers (1983) examined Salmonella-specific DNA sequences until they found certain chromosomal sequences that hybridized to Salmonella isolates at or near 100% of the time and did not cross-hybridize to E. coli or other bacteria. They were successful in detecting the presence of Salmonella in 10 different spiked food samples using DNA-DNA hybridization (Fitts et al., 1983).

DNA colony hybridization also has been used to detect and enumerate enterotoxigenic E. coli (Hill et al. 1983a) and virulent Yersinia enterocolitica (Hill et al., 1983b) in spiked scallops. The toxigenic properties and virulence factor of these bacteria are carried on plasmids, making the task of constructing a probe much simpler.

Biotechnology using recombinant DNA holds great promise for researchers who are manipulating bacteria to produce proteins and other compounds in abundance. Although in nature plasmids may occur as low as only one copy per cell (low copy number), through genetic engineering, copy number can be amplified to increase the production of the compound for which the plasmid encodes. Other research utilizing plasmids includes the splicing of genes for the production of human insulin (Crea et al., 1978) and somatostatin, a peptide hormone, (Itakura et al., 1977) by E. coli. Two proteins now being sold which were produced by recombinant DNA techniques are interferon and a vaccine for heptitis B (Baum, 1987). Current research includes using genetically engineered organisms for use in soil around wheat seedlings (Hatfield, 1987); for the production of tissue plasminogen activator (t-PA), an enzyme that

CHAPTER III

MATERIALS AND METHODS

1. Test Organisms

Four strains of P. fluorescens (P26, T25, B52, and RM14) were obtained from Dr. R. C. McKellar of the Food Research Institute, Ottawa, Canada. Of the four, only one did not produce a protease, RM14. Prior to use, strains were subcultured twice in Trypticase Soy Broth (TSB; BBL, Cockeysville, MD) with 2% (w/v) rehydrated non-fat dry milk, NDM, overnight at 25° C (Patel et al., 1983).

2. Procedures

Protease Assay

Each of the four strains of P. fluorescens was incubated in 30 ml TSB with 2% NDM at 25° C for 24-48 h. Cells were removed by centrifugation at 5,000 x g for 30 min at 4° C. The supernatant was sterilized by filtration through 0.45 u membrane filters. Ten microliters of the filtrate were placed in 6 mm diameter wells (Christen and Marshall, 1984) cut in casein digest agar (Appendix; Mayerhofer et al., 1973). Thimerosal (Sigma, St. Louis, MO) had been added to the medium at a concentration of 0.05% (w/v) to prevent the growth of bacteria. Plates containing the filtrate of strains P26, T25, and RM14 were incubated at 37° C for 48 h while those of B52 were incubated at 42° C for 48 h. Zones of proteolysis were evidenced by clearing of the medium around the wells. Ten microliters of 0.31 mM phosphate buffer, pH 7.2, were placed in one well per plate as a negative control.

Determination of the Presence of Plasmids

Microorganisms. For the plasmid preparations, pure cultures of P. fluorescens strain were grown in TSB with 2% NDM for 48 h at 25° C.

Escherichia coli V517, obtained from Dr. S. F. Barefoot, Clemson University, Clemson, S.C., was used as a positive reference strain. E. coli was reported to exhibit eight plasmids ranging in size from 1.36-35.8 megadaltons (Macrina et al., 1978). It was grown in L-broth for E. coli, (Appendix; Gerhardt et al., 1981) overnight at 37° C. Pseudomonas putida NAH^+, obtained from Dr. Gary Sayler, University of Tennessee, Knoxville, TN, also was used as a positive reference since it contains a low copy number, high molecular weight naphthalene utilization plasmid (Sayler et al., 1985). The organism was maintained on minimal medium exposed to naphthalene vapors (Appendix) and was inoculated into L-broth for Pseudomonas (Appendix; Kado and Liu, 1981) the day before use. Pseudomonas aeruginosa RM 273, also obtained from Dr. Sayler, was used as a negative reference. This strain had never exhibited plasmids (Saye et al., 1987). P. aeruginosa also was grown in L-broth for Pseudomonas overnight at 25° C.

All strains used were subcultured in their respective broth twice before each experiment. Cells were separated by centrifugation at 4,300 x g for 30 min at 4° C.

Plasmid Detection. Four procedures were used to determine the presence or absence of plasmids across the four P. fluorescens strains. These methods were derived from Anderson and McKay (1983), Kado and Liu (1981), Birnboim and Doly (1979) and a modified version of Ish-Horowicz and Burke (1981).

All solutions in the procedure of Anderson and McKay (1983) were added per 100 ml culture. The procedure involved cellular lysis in 5 ml of 6.7% (w/v) sucrose-50 mM Tris [hydroxymethyl] aminomethane hydrochloride (Tris HCl)-1 mM EDTA, pH 8.0, and 1.25 ml of 10 mg/ml lysozyme in 25 mM Tris HCl, pH 8.0 at 37° C which was a modification of the method of Klaenhammer et al. (1978). Alkaline denaturation-renaturation steps included the additions of 625 ul of 0.25 M EDTA-50 mM Tris HCl, pH 8.0 and 375 ul of 20% w/v SDS (Sigma) in 50 mM Tris HCl-20 mM EDTA, pH 8.0 at 37° C, then mixing for 30 sec. This was followed by the addition of 400 ul of fresh 3.0 N NaOH and 650 ul of 2.0 M Tris HCl, pH 7.0. Then 1 ml of 5.0 M NaCl was added to the solution before an extraction with 10 ml salt-saturated buffered phenol (Appendix) and centrifugation at 7,600 x g for 20 min at 4° C. Chloroform-isoamyl alcohol (24:1 v/v) extractions (10 ml) then were used to remove impurities, leaving the plasmid DNA in the upper phase. The plasmid DNA was precipitated from the supernatant in 2 volumes of 95% (v/v) ethanol overnight at -20° C, collected by centrifugation at 7,600 x g for 30 min at 4° C and resuspended in 10 mM Tris HCl-1mM EDTA, pH 7.5 (TE buffer).

The method of Kado and Liu (1981) employed 3 ml broth culture to which 1 ml running buffer (Appendix) and 2 ml of an alkaline SDS solution of 3% (w/v) SDS in 50 mM Tris HCl, pH 12.6, were added. This was followed by incubation at 50-65° C for 20 min to denature the chromosomal DNA. Two volumes of 1:1 (v/v) phenol-chloroform using unbuffered phenol were added to lower the pH and remove cellular debris. The phases were separated by centrifugation at 4,300 x g for

15 min at 4° C leaving plasmid DNA in the upper phase. Thirty-five microliters of the upper phase from the last centrifugation steps were mixed with tracking dye (Appendix) before application to an electrophoretic gel.

The procedure of Birnboim and Doly (1979) took advantage of the fact that linear DNA (chromosomal) will denature in a narrow pH range, 12.0-12.5, leaving covalently closed circular DNA (plasmid) unharmed. Lysis of the cells from 1.5 ml broth was achieved by holding the cells on ice for 30 min in 100 ul of 2 mg/ml lysozyme in 50 mM glucose-10 mM cyclohexane diamine tetraacetae (CDTA)-25 mM Tris HCl, pH 8.0. This was followed by the addition of 200 ul of 0.2 N NaOH-1% (w/v) SDS after which the cells were held on ice for 5 min. The pH of the preparation was reduced with 150 ul of 3M sodium acetate, pH 4.8, and the mixture held on ice for 1 h. This allowed for the formation of an insoluble clot of high molecular weight chromosomal DNA which was collected by centrifugation for 5 min at 12,800 x g in a microcentrifuge leaving the plasmid DNA in the supernatant. The plasmid DNA was removed from the supernatant by precipitation with ethanol overnight at -20° C followed by centrifugation and resuspension in 100 ul of 0.1 M Na acetate-0.05 M Tris-HCl, pH 8.0.

The modified procedure of Ish-Horowicz and Burke (1981) was similar to that of Birnboim and Doly (1979). Broth cultures (25 ml) were used. One milliliter of the first solution, 4-5 mg/ml lysozyme in 50 mM glucose-25mM Tris HCl (pH 8.0) -10 mM EDTA (pH 7.5), was added to the cells and held at room temperature 5 min. Then 2 ml of 0.2 N

NaOH-1% (w/v) SDS were added and the cells were held on ice 5 min as in the previous procedure. The pH of the solution was lowered with 1.5 ml of 3 M potassium acetate, pH 6.0, and held on ice 5 min. Chromosomal DNA was removed by centrifugation at 4,300 x g for 5 min at 4° C. The supernatant containing the plasmid DNA was extracted first with 4 ml of a 1:1 mixture of buffered phenol:chloroform, the phases separated by centrifugation for 2 min, then the upper phase was extracted with 2 ml chloroform alone and the phases again separated by centrifugation. The DNA was precipitated in ethanol for 2 min at room temperature and collected by centrifugation. The DNA pellet was washed with 70% (v/v) ethanol and then resuspended in TE buffer.

Electrophoresis. In the procedures of Anderson and McKay (1983) and Ish-Horowicz and Burke (1981), the sample in TE buffer was mixed with 1/10 volume of 1 mg/ml RNase (Sigma) and 10 ul tracking dye before application to a 0.7% (w/v) agarose gel for electrophoresis (DNA Sub Cell, Bio-Rad Laboratories, Richmond, CA). The plasmid standards *E. coli* V517 and *P. aeruginosa* RM 273 were prepared in the same fashion as the samples and applied to each gel as positive and negative controls respectively. DNA was separated by gel electrophoresis at 80 V, for 4 h using 1,500 ml running buffer (Appendix) unless otherwise specified. Ethidium bromide (Sigma) was incorporated into the gel and the running buffer at a concentration of 0.5 ug/ml for ease of visualization under an ultraviolet light (Ultra-violet Products, Inc., San Gabriel, CA). Gels were photographed (Fotodyne, Inc., New Berlin, WI) through a red filter using Polaroid Type 55 Land Film.

Antibiotic Resistance

Each of the P. fluorescens strains was grown in TSB with 2% NDM overnight at 25° C. Before plating, each of the cultures was compared to a $BaCl_2$ turbidity standard (Appendix) for inoculum density and diluted with 0.1% (w/v) peptone if necessary. Each P. fluorescens strain (0.1 ml) was spread plated onto Mueller Hinton Agar (Difco, Detroit, MI). Antibiotic disks (Table 1) were placed on the agar surface at least 24 mm apart and plates were incubated at 25° C for 48 h. The diameters of the zones were measured in millimeters and the degree of inhibition was interpreted by standards listed in Difco (NCCLS, 1984).

Attempted Transformation of RM14

A standard curve was developed to determine when cells of P. fluorescens RM14 had reached an absorbance at 500 nm (A_{550}) equivalent to 5×10^7 cells/ml. P. fluorescens RM14 initially was grown in L-broth for Pseudomonas overnight at 25° C. One milliliter was transferred to 100 ml L-broth for Pseudomonas and shaken at 100 strokes/min at 25° C for 8 h. At 1 h intervals, aliquots of the sample were removed and spread plated onto casein digest agar. At the same time, the A_{550} was determined for the cells plus medium on a Double Beam Spectrophotometer (Shimadzu UV-190, Kyoto, Japan).

Transformation experiments involved two major steps: creation of competent cells and preparation of DNA for transformation. The DNA used was prepared by the method of Anderson and McKay (1983). Aliquots of either 50 or 100 ul of the DNA resuspended in TE buffer were added to

Table 1. Antibiotics Used For Screening Pseudomonas fluorescens Strains by Disk Assay on Mueller Hinton Agar

Antibiotic	Concentration (ug)
Ampicillin	10
Amoxicillin/Clavulanic Acid	20/10
Erythromycin	15
Gentamycin	10
Lincomycin	2
Neomycin	30
Penicillin	10[a]
Streptomycin	10
Sulfisoxazole	300
Tetracycline	30

[a]Units.

the competent cells. These experiments were done to determine if protease production could be conferred on recipient protease-negative strains, such as P. fluorescens RM14.

The procedures for achieving competency and transformation are modified from Maniatis et al. (1982), Lederberg and Cohen (1974), and Cohen et al., (1972). Competency was achieved by growing P. fluorescens RM14 in L-broth for Pseudomonas until A_{550} = 0.3, which corresponded to approximately 5 x 10^7 cells/ml. Three milliliters of broth culture were removed, chilled on ice for either 10 min or 1 h, cells harvested by centrifugation at 4,000 x g for 5 min at 4° C and the pellet resuspended in 1.5 ml cold 50 mM $CaCl_2$, 10mM Tris HCl, pH 8.0. The suspension was held on ice 15 min. After a second centrifugation, the pellet was resuspended in 0.2 ml cold 50 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0. Chilled plasmid DNA from either T25, P26, or B52 was added to the competent cells and the solution held on ice 30 min. The cells were subjected to a heat pulse of 42° C for 2 min to facilitate uptake of plasmid DNA, diluted in 1.0 ml L-broth for Pseudomonas and incubated at 25° C for 30 min. Dilutions were made in 0.31 mM phosphate buffer and plated directly onto casein digest agar plates. Transformants were indicated by zones of clearing on the casein digest agar.

The quanity of DNA in each DNA preparation used was determined spectrophotometrically using the ratio of the readings at 260 nm and 280 nm (Maniatis et al., 1982). An A_{260} of 1 corresponded to 50 ug/ml DNA.

Several controls were used in an attempt to eliminate any error in interpretation of results. These controls included:

a. P. fluorescens RM14 with DNA from E. coli HB101 which contained plasmid pBR322 (obtained from Dr. G. A. Baumbach, University of Tennessee, Knoxville). This was plated on L-agar for Pseudomonas containing 25.0 ug/ml tetracycline (Sigma) as a positive control for competency and gene expression.

b. P. fluorescens RM14 with DNA from P. putida NAH^+. This was plated on minimal medium with naphthalene vapors as a positive control for incorporation of large plasmid DNA and gene expression.

c. P. fluorescens RM14, T25, P26, B52 and P. putida NAH^+ which received $CaCl_2$ treatment and TE buffer but no DNA. These strains were plated on casein digest agar to determine if the cells survived the treatment, if T25, P26 and B52 retained the ability to produce protease and if NAH^+ retained its ability to use naphthalene vapors as a carbon source. The strains also were plated on L-agar with tetracycline as negative controls.

d. P. fluorescens RM14, T25, P26 and B52 were diluted in 0.31 mM phosphate buffer and plated directly onto casein digest agar as negative and positive controls for the production of the protease.

e. *P. fluorescens* RM14 plated directly on casein digest agar, minimal medium exposed to naphthalene vapors, and L-agar with tetracycline as negative controls. The microorganism was diluted 1:1,000,000 in 0.31 mM phosphate buffer to prevent the carryover of nutrients in the broth to minimal and tetracycline media.

Eight experiments were completed and presumptive transformants were isolated and incubated in L-broth for *Pseudomonas*. Plasmid preparations by the procedure of Anderson and McKay (1983) were applied to an electrophoretic gel and plasmid profiles were compared to the profiles of the original bacteria.

CHAPTER IV

RESULTS AND DISCUSSION

1. Protease Assay

As previously outlined, cultures of P. fluorescens B52, T25, P26 and RM14 were incubated at 25° C for 24 h, pelleted by centrifugation and the supernatant filter-sterilized. The filtrate, containing the extracellular proteases, was added to wells in casein digest agar and incubated at 37° C for 48 h. Zones of clearing around the wells were considered evidence of proteolysis of the medium. Under these conditions, zones of clearing were detected only with strains P26 and T25 (Table 2). RM14 was reported to be protease negative (Torrie et al., 1983) and did not produce any clear zones. In contrast to P26 and T25, filtrate from B52 produced clear zones only after incubation of the bacterium at 25° C for 48, 72 or 96 h. The plates containing the enzyme also had to be incubated at 42° C (Table 2). With B52, zones were slightly larger when the culture had been grown for 72 and 96 h than at 48 h. To insure that the genes for protease production had been activated in strains B52, P26 and T25, all P. fluorescens cultures were grown for at least 48 h before use in plasmid preparations.

2. Plasmid Isolation and DNA Preparation

For isolation of plasmids four procedures were investigated. Their efficiency was measured using E. coli V517, a strain reported to harbor 8 plasmids (Macrina et al., 1978). Gel electrophoresis was used as the method of separation of the DNA.

Table 2. Proteolysis, as Exhibited by Zones of Clearing in Casein Digest Agar, by P. fluorescens Strains RM14, P26, T25 and B52 Incubated at 25° C

Strain	Incubation Time of Culture (h)	Size of Clear Zone (mm)[a]
RM14[b]	48	0
P26[b]	24	2
T25[b]	24	1.5
B52[c]	48	1.5

[a]Zone is measured from edge of 6 mm well to edge of clear zone
[b]Zones of proteolysis measured after 48 h at 37° C
[c]Zones of proteolysis measured after 48 h at 42° C

The procedure of Kado and Liu (1981) proved to be one of the least amenable to preparing E. coli plasmid DNA for gel electrophoresis. Gels containing plasmid preparations from this procedure exhibited few or no fluorescent bands (data not shown). This method was designed for use with a number of bacteria, including members of the family Enterobacteriaceae and a variety of plant pathogens. The procedure was developed to isolate DNA rapidly from a small broth culture (3 ml). There are several possible explanations for the results obtained. First, in achieving a rapid method, the researchers have eliminated any centrifugation and extraction steps they deemed unnecessary and time-consuming. In doing so, they may have removed an essential step. Second, the absence of lysozyme in this method may have caused insufficient lysis of cells. Third, the unbuffered phenol used in the procedure (pH 2.6) may hydrolyze the DNA in the sample to nucleotides. Fourth, any DNA in the supernatant was not concentrated by precipitation with ethanol but was applied directly to the gel. The quantity applied may have been too dilute, especially if the plasmid was in low copy number, to be detected by gel electrophoresis. Finally, the heating step diminished the amount of chromosomal DNA seen on a gel. If the plasmid DNA of E. coli V517 was too dilute to be observed and the chromosome was eliminated, no DNA remained to fluoresce on the gel.

Gels containing preparations from the method of Birnboim and Doly (1979) also exhibited no fluorescent bands in E. coli V517 (data not shown). This procedure was designed primarily to screen rapidly a

number of samples. The researchers explained that crude plasmid preparations can be examined quickly by gel electrophoresis to determine the presence of plasmids. This procedure did not employ extractions with phenol or chloroform which are commonly used to remove impurities such as proteins and help separate the aqueous and organic phases. This step was omitted because most of the protein-SDS complexes and high molecular weight RNA should have been precipitated from the supernatant by the addition of sodium acetate and collected in the centrifugation step. The absence of this step was the only difference between this procedure and that of the modified Ish-Horowicz and Burke.

The chromosome band of E. coli V517 and RNA, when RNase was not used, were detected in DNA preparations produced by the modified procedure of Ish-Horowicz and Burke (1981) and electrophoretically separated on agarose gels. No plasmid bands were seen in E. coli V517 or with any strains of P. fluorescens (data not shown). This procedure contains a delicate step using 70% ethanol to wash salt from the sides of the tube. If the ethanol is not exactly 70%, or if extreme care is not taken, some of the pellet may be lost, greatly reducing the amount of sample applied to the gel.

The procedure of Anderson and McKay (1981) consistently produced E. coli V517 plasmid preparations which were detectable by gel electrophoresis. It is thought that this procedure handles the cells and their DNA in a more gentle fashion, allowing for the concentration of the plasmid DNA without disrupting or destroying it. This protocol required neither heat nor extreme pH levels. Although this method was designed for the lactic streptococci, it had been recommended for use

with a low copy number, high molecular weight plasmid from P. putida strains (Sayler and Pettigrew, 1987). When DNA from E. coli V517 was prepared by this procedure and separated by gel electrophoresis for 3 h at 80 V, more than the eight distinct reference plasmids were detected (Figure 1, lanes 9 and 10). The reason that 10-11 bands were observed could have been due to two forms of a plasmid, supercoiled and nicked, migrating at slightly different speeds. The 3.7 megadalton band has been reported to separate into two closely migrating but discrete bands (Macrina et al., 1978). Because of the consistent results achieved with this procedure, it was used for all following experiments. Unless otherwise indicated, 100 ml was used as the culture volume. The DNA was resuspended in 200 ul of TE buffer with 1/10 volume RNase. Duplicate samples consisting of 10 ul DNA in TE buffer mixed with 10 ul tracking dye were loaded into 2 wells per strain. The concentration of the DNA was not assayed. The plasmid negative strain P. aeruginosa RM 273 also was prepared by this method using 100 ml culture incubated at 37° C for 48 h. The DNA was resuspended and loaded into gels as previously described.

None of the strains of P. fluorescens exhibited any plasmid bands by gel electrophoresis. Since pseudomonads often contain extremely large plasmids such as the 200 megadalton Inc P-2 plasmids discussed by Fennewald et al. (1978), the P. putida NAH^+ strain was used as a positive control for large plasmid isolation. It contains the high molecular weight plasmid coding for the ability to utilize naphthalene as a sole source of carbon. Normally, the plasmid is present in numbers as low as one copy per cell (Yen and Gunsalus, 1982). To compensate for

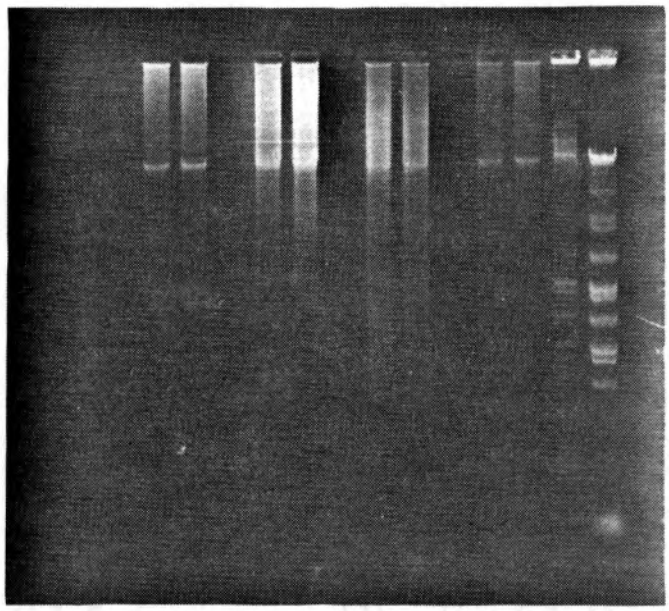


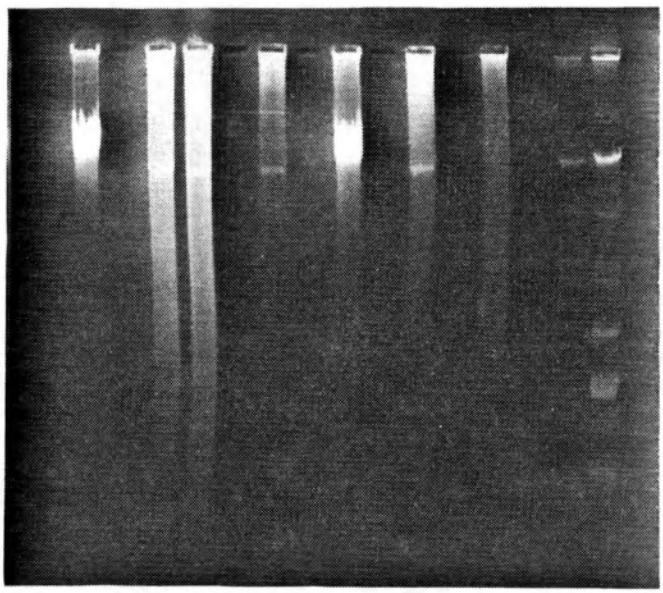
Figure 1. Electrophoretic Analysis of P. fluorescens strains for Detection of Plasmids.

Pseudomonas fluorescens strains P26, RM14, T25 and B52 were cultured for 48 h and DNA isolated as described in the text. DNA was separated in a 0.7% (w/v) agarose gel. Shown is the ethidium bromide stained electropherogram. As stated in the text, 10 ul of DNA in TE buffer were loaded in duplicate into wells 1 and 2 (P26), wells 3 and 4 (RM14), wells 5 and 6 (T25) and wells 7 and 8 (B52). Lane 9 contained 10 ul of DNA in TE buffer from E. coli V517 prepared at the same time as the P. fluorescens strains. Lane 10 contained 10 ul of DNA in TE buffer prepared previously. The molecular weights of the plasmid DNA from E. coli V517 are indicated in megadaltons in the right hand margin; chr signifies the migration position of bacterial chromosome DNA.

this low copy number, 500 ml of broth culture were prepared for plasmid isolation and gel electrophoresis. The DNA was resuspended in 1 ml TE buffer with 1/10 volume of RNase. Duplicate samples of NAH+ were loaded into the wells as previously described. The DNA from the P. fluorescens strains also was prepared from 500 ml of culture. For the P. fluorescens, 10 ul of DNA in TE buffer mixed with 10 ul of tracking dye were loaded into 1 well each. When DNA containing the NAH+ plasmid was separated by gel electrophoresis at 40 V for 10 h, the plasmid appeared to be approximately the same molecular weight as the largest E. coli V517 plasmid, 35.8 megadaltons (Macrina et al., 1978) (Figure 2, lanes 2 and 3). Electrophoresis of this gel continued for an additional 6 1/2 h at 40 V to determine if any other large molecular weight plasmids present in the wells could be detected. No more plasmid bands were found in any strain (data not shown).

3. Antibiotic Resistance

Since antibiotic resistance is often plasmid encoded, all four P. fluorescens strains were screened for their resistance to a variety of antibiotics. Susceptibility is defined as no growth by the strain in the zone of inhibition around the antibiotic disk. Since the diameter of the zone of inhibition that specifies susceptibility differs for each antibiotic, all are listed in Table 3 (NCCLS, 1984). Resistance of the strains to each antibiotic was defined as growth greater than or equal to the specified zone diameter as listed by NCCLS (1984). These zones also differ for each antibiotic and are listed in Table 3. Growth of the strain between these two zone diameters was considered intermediate resistance.



1, 2,3 4, 5, 6, 7, 8,9

Figure 2. Electrophoretic Analysis of P. fluorescens Strains for Detection of Plasmids.

Pseudomonas fluorescens strains P26, RM14, T25 and B52 were cultured for 48 h and DNA isolated as described in the text. DNA was separated in a 0.7% (w/v) agarose gel. The ethidium bromide stained electropherogram is shown. Lane 1 contained 10 ul of DNA in TE buffer from 100 ml culture of P. aeruginosa RM 273 prepared as described in the text. Lanes 2 and 3 are duplicates and contained 10 ul of DNA in TE buffer from P. putida NAH+. As stated in the text, 10 ul of DNA in TE buffer were loaded into well 4 (P26), well 5 (RM14), well 6 (T25) and well 7 (B52). Lane 8 contained 10 ul of DNA in TE buffer from 500 ml of culture of E. coli V517 prepared at the same time as the P. fluorescens and P. putida. Lane 10 contained 10 ul of DNA in TE buffer prepared previously. Plasmid denotes the migration position of the large molecular weight plasmid of E. coli V517 and P. putida NAH+.

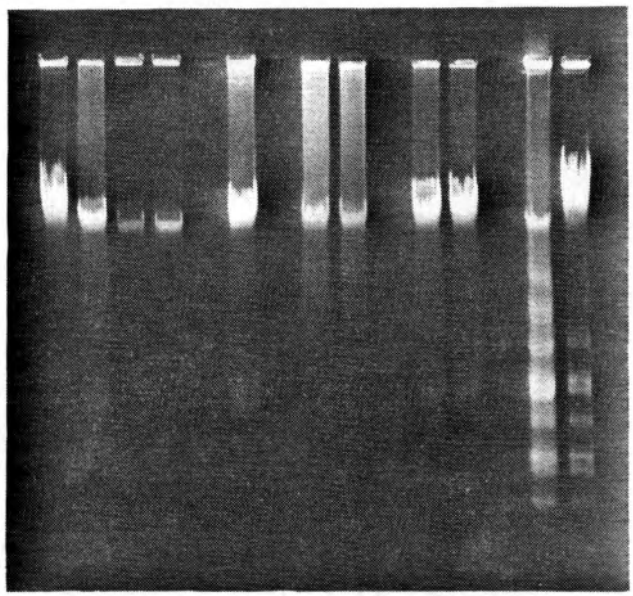
Table 3. Antibiotic Resistance of P. fluorescens Strains RM14, T25, P26 and B52 Measured by Zones of Clearing Around Each Antimicrobic Disk After Incubation at 25° C for 48 h

Antibiotic	P. fluorescens strains					
	RM14	T25	P26	B52	Susc[a]	Res[b]
	mm					
Amoxicillin/ Clavulanic Acid	0	0	0	0	18	13
Ampicillin	0	0	0	0	14	11
Erythromycin	0	0	0	0	18	12
Gentamycin	21	14	18	21	15	13
Lincomycin	0	0	0	0	N/A[c]	N/A
Neomycin	20	15	17.5	20	17	12
Penicillin G	0	0	0	0	22	11
Streptomycin	16	16	16	16	15	11
Sulfisoxazole	0	0	0	0	17	12
Tetracycline	15	14.5	13.5	16	19	14

[a]Susceptibility-Strain is susceptible if zone is equal to or greater than number indicated (NCCLS, 1984).
[b]Resistance-Strain is resistant if zone is equal to or less than number indicated (NCCLS, 1984).
[c]Not available.

The four strains exhibited similar patterns (Table 3). For example, all were either in the intermediate range of susceptibility or were completely susceptible to streptomycin, gentamycin, tetracycline and neomycin at 24 h. However, after 48 h, small satellite colonies appeared within the zones of inhibition around the streptomycin disks. This was true for all four strains. Since antibiotic resistance often is encoded on a plasmid, these satellite colonies were picked and prepared for plasmid isolation. One representative colony of each strain was picked from within the zones, plated on Mueller Hinton Agar and streptomycin disks applied to the medium. All strains except B52 exhibited resistance to streptomycin. The resistance of T25 to penicillin and susceptibility to streptomycin was in accordance with the results of Patel et al. (1983).

To determine if the streptomycin resistant colonies contained plasmids, one colony was picked from each plate of T25, P26, RM14 and a colony each of T25 from around the exythromycin disk and the ampicillin disk. The streptomycin resistant colonies were designated T25-Str+, P26-Str+, and RM14-Str+. For convenience, the erythromycin and ampicillin colonies were pooled, grown together and designated T25-Ery+/Amp+. These colonies were grown in TSB plus 2% NDM for 48 h and plasmid preparations prepared according to the procedure of Anderson and McKay (1983). No plasmid DNA bands were detected in the lanes of P26, T25-Str+ and T25-Ery+/Amp+ (Figure 3). No discrete plasmid bands were seen. The indistinct lines are thought to be evidence of chromosomal degradation.



1,2,3,4 5, 6,7 8,9 10,11

Figure 3. Electrophoretic Analysis of P. fluorescens Strains for Detection of Plasmids.

Pseudomonas fluorescens strains P26, RM14, T25 and B52 were cultured for 48 h and DNA isolated as described in the text. DNA was separated in a 0.7% (w/v) agarose gel. Shown is the ethidium bromide stained electropherogram. All lanes contained 10 ul DNA in TE buffer from 100 ml culture prepared as described in the text. Lane 1 contained DNA from P. aeruginosa RM 273. Lane 2 contained DNA from P. fluorescens strain P26. Lanes 3 and 4 contained duplicate DNA samples from P26 resistant to streptomycin. Lane 5 contained DNA from T25 resistant to streptomycin. Lanes 6 and 7 contained duplicate DNA samples from RM14 resistant to streptomycin. Lanes 11 and 12 contained DNA from T25 resistant to erythromycin and ampicillin. Lane 10 contained DNA from E. coli V517 prepared at the same time as the P. fluorescens strains. Lane 11 contained DNA from E. coli V517 prepared previously.

Since all strains were either in the intermediate range or were susceptible to tetracycline, it was used as a marker for the transformation experiments. E. coli HB101 contained plasmid pBR322 which carries resistance genes for ampicillin and tetracycline. It was used as a positive control for competency and gene expression.

3. Transformation

A graph of A_{550} versus log CFU/ml was developed to determine what reading at an absorbance of 500 nm (A_{550}) correlated with a cell density in the range of 5×10^7-1×10^8 cell/ml of the recipient strain, RM14. Plates of casein digest agar which had been spread with 0.1 ml culture in duplicate every hour for 8 h were counted after 48 h of incubation at 25° C. The A_{550} of the culture was taken at each plating. Absorbance readings at 550 nm between 0.26-0.3 corresponded to the desired concentration of cells (Figure 4). Therefore, RM14 was grown to an A_{550} between 0.26 and 0.3 before use in each experiment so that the cells always would be in the logarithmic phase of growth.

In the first experiment, the 3 ml broth culture was held on ice 10 min before centrifugation. In all of the following experiments the broth cultures were held on ice 1 h before centrifugation. Maniatis et al. (1982) reported that in the procedure for E. coli no more than 100 ul DNA suspended in TE buffer from 3 ml broth should be added to the recipient cells, therefore all DNA that was added to the cells was in quantities less than 100 ul. Most frequently either 100 ul and 50 ul of DNA were added. Before plating, dilutions of competent cells were made

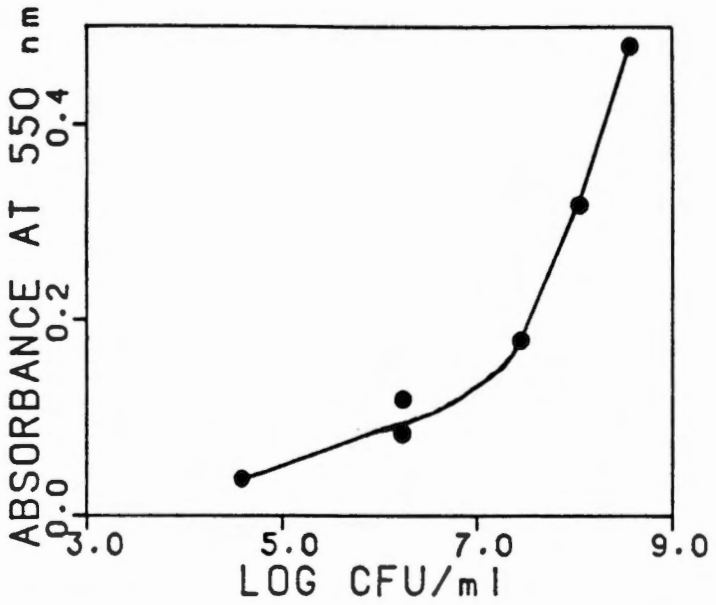


Figure 4. Absorbance at 550 nm versus the log CFU/ml of P. fluorescens RM14.

Pseudomonas fluorescens RM14 was grown for 8 h at 25° C as described in the text. At 1 h intervals, 0.1 ml was spread plated in duplicate onto casein digest agar. At the same time the A_{550} was determined for the cells plus medium. Circles denote actual readings at 550 nm that correspond to the mean of the log CFU/ml from duplicate plates incubated for 48 h at 25° C. A_{550} values between 0.26 and 0.3 correspond to the range of $5 \times 10^7 - 1 \times 10^8$ cells/ml.

in 0.31 mM phosphate buffer for three reasons: 1) to give plates with colonies between 25-250 CFU/ml, 2) to detect zones of proteolysis more clearly and 3) to eliminate the possibility that RM14 could utilize nutrients carried over from the L-broth to the tetracycline and minimal medium, thereby giving false positives.

Two types of controls for recipient RM14 were used, plates of a 1:1,000,000 dilution of RM14 in 0.31 mM phosphate buffer and plates of RM14 which had received the treatment for transformation but to which TE buffer had been added without DNA. Both control plates of RM14 on minimal medium exposed to naphthalene vapors exhibited approximately 75 pinpoint colonies. Control plates of RM14 were not expected to grow on minimal medium since a plasmid codes for the ability to use naphthalene and no plasmids were seen in previous electropherograms containing RM14 DNA. When RM14 received plasmid DNA from P. putida NAH+, it produced approximately 500 colonies. However, since RM14 had the ability to grow on minimal medium and use naphthalene as a sole carbon source, there was no way to determine visually which colonies might be transformants and which colonies already had the ability to grow using naphthalene as a carbon source. The only way to distinguish between the true transformants and the spontaneous mutants would be to prepare each colony for plasmid isolation and gel electrophoresis. This was not done. Therefore, we are not confident that competence had been achieved and the incorporation of large plasmid DNA is possible.

No transformation was seen using RM14 as a recipient for plasmid pBR322 which confers tetracycline resistance. When RM14 was incubated with 1.45 ug or 1.45 ng E. coli HB101 DNA with the pBR322 plasmid, no

transformants were detected by growth in the presence of 25 ug/ml tetracycline. E. coli HB101 with pBR322 was capable of growing on agar containing tetracycline incorporated at this level.

Transformation experiments have been designed for use in E. coli and may not be applicable to other genera of bacteria without certain considerations. The procedures used may not have made P. fluorescens competent for uptake of pseudomonad plasmid DNA. Results using NAH+ plasmid DNA from P. putida for uptake by P. fluorescens RM14 were inconclusive, and transformation was not confirmed by gel electrophoretic analysis of DNA. Pseudomonas fluorescens may contain nucleases which recognize and destroy the foreign E. coli plasmid, pBR322. However, even if the DNA from E. coli is taken up by P. fluorescens and is not destroyed upon entry to the cell, the P. fluorescens may not have the capability to express the foreign genes. For P. fluorescens to take up and express genes from E. coli efficiently, certain modifications of the procedure may need to be undertaken.

Unlike transformation experiments where antibiotic resistance is expressed by the transformant and is exhibited by growth on the antibiotic plate, growth of P. fluorescens always was present on the casein digest agar. Transformants are defined as RM14 colonies that exhibit proteolytic activity (produce clear zones) around the colony. Clear zones always extended at least 2 mm from the edge of the colony.

Eight experiments were completed using RM14 as the recipient of DNA from the three other P. fluorescens strains. DNA from three separate preparations of T25 were used and each is designated in Table 4. The DNA from only one preparation of P26, B52 and RM14 was used.

Table 4. Transformation Experiments Using P. fluorescens RM14 as the Recipient Strain for Plasmid Preparations from P. fluorescens T25, P26, B52 and RM14

Expt.	Mass Added	DNA Source	Mean No. Colonies	Mean Protease Positive Colonies	No. Transformants/ ug
I	ND[a]	T25 (Prep. 1)[b]	272	0	ND
		Treated R14[c]	226	0	ND[d]
		Untreated RM14[e]	248	0	NA
II	ND	T25 (Prep. 1)	300	290	ND
		Treated RM14	282	0	NA
		Untreated RM14	316	0	NA
III	ND	T25 (Prep. 1)	no colonies	-	--
	0.58 ug	P26	2.2×10^4	0	0
	0.81 ug	B52	1×10^4	0	0
		Treated RM14	4.1×10^3	0	NA
		Untreated RM14	286	0	NA
IV	ND	T25 (Prep. 1)	314	7	ND
	0.58 ug	P26	1.4×10^4	4.3	7.3
	0.81 ug	B52	1.1×10^4	2.3	2.8
		Treated RM14	2.0×10^4	2	NA
		Untreated RM14	270	0	NA
V	ND	T25 (Prep. 1)	314	7	ND
	0.58 ug	P26	2×10^3	8	137.9
	0.81 ug	B52	524	31	382.7
		Treated RM14	1.1×10^3	0	NA
		Untreated RM14	252	0	NA
VI	2.59 ng	T25 (Prep. 2)	388	0	0
	0.58 ng	P26	2×10^4	0	0
	0.81 ng	B52	5.2×10^3	0	0
		Treated RM14	416	0	NA
		Untreated RM14	106	0	NA

Table 4. (Continued)

Expt.	Mass Added	DNA Source	Mean No. Colonies	Mean Protease Positive Colonies	No. Transformants/ ug
VII	2.59 ug	T25 (Prep. 2)	356	0	0
	0.58 ug	P26	5	0	0
	0.81 ug	B52	5	0	0
	0.7 ug	RM14	31	0	0
	2.59 ng	T25 (Prep. 2)	71	0	0
	0.58 ng	P26	10	0	0
	0.81 ng	B52	26	0	0
	0.7 ng	RM14	187	0	0
		Treated RM14	310	0	NA
		Untreated RM14	142	0	NA
VIII	2.59 ug	T25 (Prep. 2)	312	0	0
	N/D	T25 (Prep. 3)	274	0	0
	0.58 ug	P26	1×10^4	0	0
	0.81 ug	B52	3.2×10^3	0	0
	0.7 ug	RM14	422	0	0
		Treated RM14	2.6×10^3	0	NA
		Untreated RM14	202	0	NA

[a]Not determined.
[b]Number of DNA preparation.
[c]RM14 with $CaCl_2$ treatment but without the addition of DNA.
[d]Not applicable.
[e]RM14 diluted 1:1,000,000 and plated directly on casein digest agar.

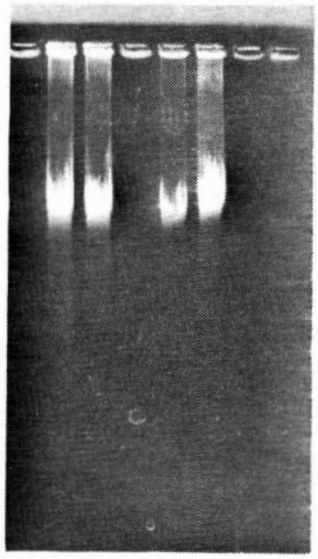
In experiments II, IV and V but not I, RM14 exhibited protease activity when DNA from T25 was added to the recipient cells (Table 4). Since the number of transformants/ug cannot be determined in these experiments, the percentage of apparent transformants was calculated by dividing the number of proteolytic colonies by the total number of colonies per plate (data not shown). In Experiments II and IV, apparent transformants were greater than 90%, however, in Experiment IV only three colonies were evident and all three produced zones of clearing. When DNA from P26 or B52 was used, consistently fewer transformants/ug were noticed. This may be due to the lower concentration of DNA isolated from these strains. Spontaneous revertants (proteolytic colonies seen by RM14 treated with $CaCl_2$ but without the addition of DNA) were noticed only in Experiment IV (Table 4).

Maniatis et al. (1982) suggested that 40 ng DNA was the maximum level for use in transformation with E. coli. In Experiments I-V 0.58 and 0.81 ug P26 and B52 of DNA respectively were added to the competent cells. After protease activity was noticed in RM14 in Experiments II, IV and V, the DNA was diluted 1:1000 in Experiments VI and VII. No protease positive colonies were obtained. Experiment VIII was an attempt to repeat the results obtained in experiments II and IV, but was unsuccessful.

Untreated RM14 never exhibited protease activity on casein digest agar. DNA from RM14 was added to RM14 in experiments VII and VIII to determine if the presence of its own genes in the cells would cause the production of the protease. No proteolysis was detected.

One random protease positive RM14 colony treated with DNA from T25 was picked from a plate in experiment II and streaked on casein digest agar. All colonies on this plate were protease positive. One random colony was prepared for gel electrophoresis as previously described and the plasmid profile compared to a protease negative strain of RM14. No plasmid bands were noticed in either strain (Figure 5, lanes 1, 2 and 3, 4). Therefore, protease activity could not be attributed to the presence of a plasmid in the range of 1.4-35.8 megadaltons as detected by gel electrophoresis.

Results were variable making interpretation difficult. Since proteolysis was exhibited by RM14 only when the cells were exposed to 1000 times the reported maximum amount of DNA as recommended for E. coli (Maniatis et al., 1982), perhaps an excess of genes from the protease positive strains is necessary to transform in Pseudomonas. It is also possible that the genes acted to reactivate the mutated genes (Torrie et al., 1983) for protease production in RM14. Another possibility was the presence of either phage DNA within the chromosome or an episome (plasmid DNA in the chromosome). The incorporation of a plasmid or phage into the chromosome may explain why no extrachromosomal bands were detected by gel electrophoresis. However, minute quantities (picogram levels) of phage or plasmid DNA released from the chromosome may go undetected by gel electrophoresis and still maintain the ability to transform. This may explain why the experiment produced protease positive colonies in Experiments II, IV and V. Although no plasmid bands were seen in the plasmid preparation of the colony from Experiment



1,2 3,4 5

Figure 5. Electrophoretic Analysis of Protease Positive P. fluorescens RM14 for Detection of Plasmids.

Both the protease positive and the protease negative strains of P. fluorescens RM14 were cultured for 48 h and DNA isolated as described in the text. DNA was separated in a 0.7% (w/v) agarose gel. The ethidium bromide stained electropherogram is shown. Lanes 1 and 2 were duplicates and contained 10 ul DNA in TE buffer from protease negative RM14. Lanes 3 and 4 contained duplicate samples of 10 ul of DNA in TE buffer from protease positive RM14. Lane 5 contained 10 ul of DNA in TE buffer from a previous preparation of E. coli V517.

II, it is possible that this was not a transformant but a spontaneous revertant. Screening a large number of protease positive colonies for the presence of a plasmid would have eliminated the possibility of choosing a spontaneous revertant. The variability between the experiments may be attributed to the age of the plasmid preparations. By the time Experiments VI, VII and VIII were completed, the plasmid preparations were between 5 and 7 weeks old. Kado and Liu (1981) report that plasmid preparations can be kept for up to six weeks at 4° C. However, considering the sensitive nature of these experiments, any degradation of the DNA may diminish the efficiency of transformation.

CHAPTER V

CONCLUSIONS

The procedure of Anderson and McKay (1981) was successful in producing DNA plasmid preparations for the standards of reference, *E. coli* V517 and *P. putida* NAH+. It also was successful in preparing DNA preparations of *P. fluorescens* RM14, P26, T25 and B52, however no plasmid bands were observed in the range of 1.4-35.8 megadaltons using gel electrophoresis as the method of detection. It is possible that these strains may contain an extremely large plasmid, similar to the 200 megadalton Inc P-2 plasmid of *P. aeruginosa*, which would not be detectable by gel electrophoresis using the present conditions. It is also possible that these strains do not harbor a plasmid.

Antibiotic resistance screening showed that all four strains of *P. fluorescens* were resistant to the same six antibiotics. However, plasmid profiles of strains grown under antibiotic stress still exhibited no plasmid bands. Tetracycline resistance as a marker for gene expression proved to be unsuitable since no transformants were observed in the presence of 25 ug/ml tetracycline.

Transformation experiments seemed to be successful only when the RM14 was exposed to 1000 times the reported maximum level of DNA. Plasmid profiles of the transformants resembled the original profile of RM14, reiterating the previous conclusion that no plasmids exist in these strains in the range of 1.4-35.8 megadaltons. Since RM14 is not inherently protease negative but is a mutant of a protease positive strain, perhaps exposing it to such great quantities of DNA from the

other protease positive strains acted as a trigger to reactivate the genes for protease production. It is also possible that transformation did occur, but that the transforming agent resides in the cell in such a small quantity that it is undetectable by gel electrophoresis and that 1000 times the reported level of DNA must be present to overcome the chromosomal contamination observed in all electropherograms.

This study clearly indicated the need for further research on the genetic regulation of the proteases of P. fluorescens. It is possible that all protease production is encoded in the chromosomal DNA, and if so, research in that area may further explain the results achieved in this study.

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APPENDIX

1. Media

A. Casein Digest Agar (Mayerhofer et al., 1973)

Standards Methods Agar	23 g/l
Skim Milk	150 ml

B. L-broth for Pseudomonas (Kado and Liu, 1981)

Casein hydrolysate	10 g/l
NaCl	10 g/l
Yeast extract	5 g/l
Agar	15 g/l

C. L-broth for E. coli (Gerhardt et al., 1981)

Tryptone	10 g/l
Yeast extract	5 g/l
NaCl	0.5 g/l

The pH was adjusted to 7.0 with 1 N NaOH and 10 ml of filter-sterilized 20% (w/v) glucose was added per liter after autoclaving.

D. Minimal Medium (Sayler et al., 1985)

$NaNO_3$	4.0 g/l
KH_2PO_4	1.5 g/l
$Na_2HPO_4.7H_2O$	0.5 g/l
$FeCl_3.6H_2O$	0.0005 g/l
$MgSO_4.7H_2O$	0.2 g/l
$CaCl_2.2H_2O$	0.01 g/l
Agar	15 g/l

The pH was adjusted to 6.8-7.0 with 2 N NaOH. Naphthalene was placed in a crucible on the bottom of a dessicator. Plates were inverted and exposed to vapors in the dessicator without vacuum at room temperature for 48 h.

E. 12.5 ug/ml Tetracycline Medium (Maniatis et al., 1982)

L-agar for Pseudomonas	1 l
Tetracycline solution	2 ml

The tetracycline solution was composed of 12.5 mg/ml tetracycline in 50% (v/v) ethanol which was dispensed by 5 ml aliquots into sterile amber vials and stored at -20° C. Plates containing tetracycline were stored in the dark at 4° C.

2. Solutions

A. Running Buffer (Kado and Liu, 1981; Maniatis et al., 1982)

Concentrated Stock Solution

Tris [hydroxymethyl] aminomethane (Tris)	96.8 g/l
Acetic acid	22.84 mls
0.5 M ethylenediaminetetraacetate (EDTA) (pH 8.0)	80 mls

The pH of final stock solution was adjusted to 7.9 with 2 N NaOH.

The working solution was 100 ml stock plus 1,900 ml double distilled water. The concentration of the working solution was 40 mM Tris-acetate-2 mM EDTA.

B. Tracking Dye (Kado and Liu, 1981)

Bromcresol purple	0.25 g
Glycerol	50 ml
0.05 M Tris-acetate (pH 7.9)	50 ml

C. Ribonuclease A (RNase) (Birnboim and Doly, 1979; Maniatis et al., 1982)

RNase	10 mg
5mM Tris-hydrochloride (Tris HCl) (pH 8.0)	10 ml

The solution was heated at 100° C and allowed to cool slowly to room temperature. It was dispensed in 1 ml aliquots and stored at -20° C.

D. Buffers for Phenol (Maniatis et al., 1982)

1.0 M Tris HCl (pH 8.0)

0.1 M Tris HCl (pH 8.0)

Frozen distilled phenol was warmed to 60° C and 8-hydroxyquinoline added to a final concentration of 0.1% (w/v). The phenol was extracted with 2 volumes of 1.0 M Tris HCl (8.0) with 0.2% (v/v) 2-mercaptoethanol. The buffered phenol then was saturated with a 3% (w/v) sodium chloride solution and stored at -20° C.

E. Barium Chloride ($BaCl_2$) Turbidity Standard (NCCLS, 1984)

1.175% $BaCl_2 \cdot 2H_2O$	0.5 ml
0.36 N H_2SO4	99.5 ml

The solution was distributed in 6 ml amounts into screw cap tubes and stored at room temperature in the dark. Tubes were agitated on a mechanical vortex before use.

VITA

Margaret Carlisle Chappell was born in Columbia, South Carolina on October 14, 1962. After graduation from Richard Winn Academy in May 1981, she entered Clemson University, Clemson, South Carolina the following August. In May 1985, she received a Bachelor of Science degree in Microbiology from Clemson University and in July of the same year she entered the University of Tennessee, Knoxville. A Master of Science degree in Food Technology and Science was awarded to her in December 1987.

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