INVESTIGATION OF THE GENETIC LOCATION OF THE PROTEASE GENES IN Pseudomonas Fluorescens STRAINS T24 AND T25

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INVESTIGATION OF THE GENETIC LOCATION OF THE PROTEASE GENES IN *PSEUDOMONAS FLUORESCENS* STRAINS T24 AND T25

BY

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Abstract

Economic and industrial interest in prokaryotic extracellular proteases has been the impetus for the study of extracellular proteases. *Pseudomonas fluorescens* strains T24 and T25, have been recognized for their ability to produce extracellular proteases, and the presence of plasmid DNA in these strains led to the hypothesis that the protease genes may be located on the plasmid DNA. To determine whether or not the protease genes were located on the plasmid DNAs of the two strains, various experiments, including conjugation, transformation, and curing via heat treatment were employed. While protease positive transconjugants and transformants were not detected in the conjugation and transformation experiments, respectively, the curing experiment suggested the plasmid location of the protease genes.

Partial restriction endonuclease digestion maps were created for the plasmids, pT24 and pT25. The single restriction endonuclease digests for each of the plasmids was then probed with a nonradioactive, digoxigenin-labeled construct, pUT 8, reported to contain a protease gene. The pUT 8 construct hybridized specifically with a 3.75 kbp *Sal* I fragment and two *Pst* I fragments (sizes 3.4 kbp and 1.72 kbp) from the pT24 and pT25 plasmids, providing a second line of evidence for the plasmid origin of the protease genes. Regions similar to the pUT8 construct located on the pT24 and pT25 plasmids were then ligated to the cloning vector pUC18, and used to transform *Escherichia coli*.

While thousands of colonies were screened, protease positive transformants were not detected. The pUT 8 probe was also used to probe the genomic DNA from *P. fluorescens* strains T24 and T25. The probe did not hybridize with digests of genomic DNA from either *Pseudomonas* strain, thus providing a third line of evidence for the plasmid origin of the protease genes.

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List of Abbreviations

%	Percentage
Ω	Ohm
~	Approximately
Amp	Ampicillin
Anti-AP	Anti-digoxigenin alkaline phosphatase
ATP	Adenosine triphosphate
Ba	Barium
bom	Basis of mobility
°C	Degrees Centigrade
$CaCl_2$	Calcium chloride
Ca^{2+}	Calcium ion
CIP	Calf intestinal phosphatase
cm	Centimeter
CTAB	Hexadecyltrimethyl ammonium bromide
DIG	Digoxigenin
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid, disodium salt
EGE	Extrachromosomal genetic elements
EtBr	Ethidium Bromide
F	Fertility factor
fin	Fertility inhibition
g	Gravity
HCl	Hydrochloric acid
hr(s)	Hour(s)
HTST	High-temperature, short-time
Inc	Incompatibility
IPTG	Isopropyl-β-D- thiogalactopyranoside
kbp	Kilobase pair

KCl	Potassium chloride
KOAc	Potassium acetate
λ	Lambda
L	Liter
lb	Pound
LB	Luria Bertani medium
LiCl	Lithium chloride
LTH	Low temperature holding
LTI	Low temperature inactivation
M	Molar
mg	Milligram
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	Minute(s)
mL	Microliter
mM	Millimolar
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
mob	Mobilization
MP	Milk powder
msec	Millisecond
NaCl	Sodium chloride
NAH	Naphthalene
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium salt
nm	Nanometer
OD	Optical density
OF	Oxidation-fermentation medium
o/n	Overnight
ori T	Origin of transfer
pDNA	Plasmid DNA

PIPES	[Piperazine-N,N'-bis(2-ethanesulfonic acid)]
Rb	Rubidium
rpm	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
sec	Second(s)
SOC	Outgrowth medium
sp.	Species
Sr	Strontium
SSC	Sodium chloride/sodium citrate
TBE	Tris-borate buffer
TE	Tris-hydrochloride EDTA buffer
TOL	Toluene
tra	Transfer
Tris-HCl	[Tris(hydroxymethyl)aminomethane)]-hydrochloric acid
TSA	Trypticase soy agar
TSB	Trypticase soy broth
U	Units
µF	Microfarads
µg	Micrograms
UHT	Ultra-high-temperature
µL	Microliter
UV	Ultraviolet light
V	Volts
x	Times
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
X-phosphate	5-bromo-4-chloro-3-indolyl phosphate

Chapter 1
General Introduction

1.1. History of the *Pseudomonas fluorescens* strains

Pseudomonas fluorescens strains T24 and T25 were isolated from raw milk, from a local dairy, and characterized in the early 1980's (Patel et al. 1983). Research indicated that the isolated bacteria were gram negative, oxidase and catalase positive, polarly flagellated rods, capable of growth in the 0° to 35°C temperature range, but which had a 20°C to 25°C temperature optimum. The various isolates were able to grow at pH values in the range of 5 to 9, with an optimal pH between 6 and 7, and were able to produce a yellowish-green pigment (phycocyanin) that fluoresced under ultraviolet (UV) light. The isolates were differentiated on the basis of various assays including susceptibility or resistance to various antibiotics, heat stability of proteases, substrate specificity of proteases and molecular weight of proteases. *P. fluorescens* strains T24 and T25 were differentiated on the basis of antibiotic susceptibility differences; strain T24 was susceptible to chloramphenicol (30 µg) and resistant to streptomycin (10 µg), while strain T25 was resistant to chloramphenicol and susceptible to streptomycin.

P. fluorescens strains T24 and T25 were both capable of producing a single extracellular protease (Patel et al. 1983). The protease produced by *P. fluorescens* strain T25 was the subject of additional research, and it was characterized as a metalloprotease with a molecular weight of 42,000 ± 1, 500 KDa (Jackman et al. 1983). Immunological assays, using antiserum produced against the *P. fluorescens* strain T25 protease, indicated that the proteases from both the T25 and T24 shared common antigenic determinants (Jackman et

al. 1983).

1.2. Psychrotrophs

P. fluorescens strains T24 and T25 are psychrotrophic bacteria. On the recommendation of Eddy in 1960, psychrotrophs were defined as bacteria that were capable of growth at 5°C or less, regardless of their optimum growth temperature (Suhren 1989). However, the currently accepted definition, adopted by the International Dairy Federation (IDF) in 1976, considers psychrotrophs to be microorganisms that can grow at 7°C or less, irrespective of their optimum growth temperature, which is usually in the mesophilic range between 20°C and 30°C (Suhren 1989; Cromie 1992; Shah 1994).

1.2.1. Proteases produced by psychrotrophs

The extracelluar proteases produced by psychrotrophs are generally classified as neutral to alkaline metalloproteases (Fairbairn and Law 1986; Kohlman et al. 1991). Metalloproteases are protein degrading enzymes that contain metal ions, such as zinc and calcium, at their active sites (Cromie 1992). The pH optima for the metalloprotease activity is generally between 6.5 and 8.0 (Fairbairn and Law 1986). Psychrotrophs are capable of producing the extracellular proteases over a wide temperature range, which includes refrigeration temperatures (0°C to 7°C), but are optimally produced in the 20°C to 30°C temperature range (Cromie 1992).

1.2.2. The effect of proteases on the dairy industry

In the past, the major spoilage microorganisms of milk were the lactic acid bacteria and other mesophilic bacteria, due to inadequate cooling of milk

(Fairbairn and Law 1986). However, in the early 1950s refrigerated bulk tanks were introduced to the dairy industry, eliminating the problems associated with the growth of mesophilic bacteria (Fairbairn and Law 1986; Garcia et al. 1989). Unfortunately, a common practice in many developed dairy industries is to extend the refrigeration storage of raw milk, up to 5 days, before processing, which selects for psychrotrophic bacteria (Stepaniak et al. 1982; Champagne et al. 1994; Azcona et al. 1988).

Psychrotrophic gram negative rods comprise a minor percentage (~ 10%) of the initial raw milk flora, but with extended storage, rapidly become the dominant flora (Shah 1994; Garcia et al. 1989). The majority of these bacterial species are *Pseudomonas*, especially *P. fluorescens* (Craven and Macauley 1992; Kohlman et al. 1991). It is during the extended refrigerated storage of the milk that the psychrotrophic bacteria produce extracelluar enzymes, specifically proteases and lipases (Cromie 1992). Low temperature holding (LTH) pasteurization (62.8°C for 30 min.), high-temperature, short-time (HTST; 71°C for 15 sec.), and ultra-high-temperature (UHT; 141°C for 2 sec.) processing of milk does destroy the psychrotrophic bacteria present, but the enzymes, especially the proteases, are very thermostable and are not inactivated by these heat treatment processes (Prescott et al. 1990; Champagne et al. 1994; Fairbairn and Law 1986).

The proteases that remain in the milk after heat processing are primarily responsible for quality problems and reduced shelf-life of dairy products (Garcia et al. 1989; Mitchell and Marshall 1989). Some of the common milk quality problems associated with the presence of proteases in milk products include: bitter and rancid flavours, browning of milk upon heating, off odours and coagulation or gelation of the milk (Champagne et al. 1994; Fairbairn and Law 1986; Shah 1994; Kohlman et al. 1991; Adams et al. 1975). The other dairy product that may be affected by the presence of these proteases is cheese.

Kohlman et al. (1991) and Hicks et al. (1982), both indicate that these enzymes decrease the cheese yield due to poor curd formation in cheese making.

1.2.3. Controlling the production of proteases in milk

The economic loss incurred by the dairy industry, due to the presence of these extracellular proteases produced by psychrotrophic bacteria, has been the impetus for research. Various nutritional factors, such as the influence of calcium (McKellar and Cholette 1986), iron (McKellar et al. 1987), metal ions (Margesin and Schinner 1992), complex media (Roussis et al. 1990), carbon (McKellar 1989) and nitrogen sources (Fortina et al. 1989), on the production of these extracellular proteases, have been studied. However, the findings have been inconclusive and contradictory. Additionally, the influence of temperature (Margesin and Schinner 1992) and pH and oxygen (McKellar 1989) on extracellular protease production have also been researched, but have not produced any conclusive findings.

Attempts to destroy extracelluar proteases produced by psychrotrophs, either by extending the holding time or increasing the temperature, have led to adverse effects, such as off flavours, in the milk and milk products (Gebre-Egziabher et al. 1980). The destruction of the proteases by low temperature inactivation (LTI), does have potential (Stepaniak et al. 1991). Low temperature inactivation is an inactivation of the enzyme at temperatures just above the optimum temperature for activity (Barach et al. 1978). At approximately 55 °C the proteases produced by a *Pseudomonas sp.* and *P. fluorescens* P1 are susceptible to inactivation, via self-digestion of the enzymes (Shah 1994; Stepaniak et al. 1991).

1.2.4. Industrial applications of proteases

Aside from the detrimental and costly effects of proteases to the dairy industry, proteases have enormous potential for industrial applications. According to Mitra et al. (1996), proteases in general are used as detergent additives, in fermentations, in food, and as depilatory agents in the leather industry. Proteases have also been used in pharmaceutical industries, as catalysts of chemical transformations, and have the potential to decrease the ripening time in the cheese making process (Rahman et al. 1993; Jakubke 1994; Jackman et al. 1985)

1.2.5. Determining the genetic location of protease genes

Knowledge of the genetic location of protease genes would provide insight into the production, regulation and secretion of extracellular proteases by psychrotrophic bacteria. Unfortunately, the genetic origin of the protease genes of psychrotrophic pseudomonads has not been extensively researched. However, extensive research has been conducted on various medically significant strains of *Pseudomonas aeruginosa* and the protease genes were found to be located on the chromosomal DNA (McKellar 1989; Gambello and Iglewski 1991). The gene for the alkaline serine protease from an alkaline-resistant *Pseudomonas sp.* was also located on the chromosomal DNA (Jang et al. 1996).

Hatfield (1990) was able to cure *P. fluorescens* strain T20 of its plasmid using the chemical N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and the cured colonies lost their protease activity, thus providing the linkage between the protease activity and a possible plasmid location for the protease gene(s). Unfortunately, MNNG causes mutations in all DNA, both chromosomal and plasmid (Potter and Loutit 1983). Consequently, more conclusive evidence is

needed to establish the genetic location of the protease gene in this particular strain of *P. fluorescens*.

1.3. Plasmids

Originally defined by Lederberg in 1952 to describe all extrachromosomal hereditary determinants (Crosa et al. 1994), plasmids are currently defined as extrachromosomal, autonomously replicating segments of DNA, that are generally double-stranded, circular molecules and stably inherited (Chakrabarty 1976; Pelczar et al. 1993; Hardy 1981; Prescott et al 1990). Frequently referred to as extrachromosomal genetic elements (EGE), plasmids are widely distributed among most prokaryotic species, and have also been found in a few eukaryotic species (Dale 1989; Pelczar et al. 1993; Crosa et al. 1994; Ohman 1988).

Believed to arise from any piece of DNA that acquires the ability to replicate independently of the chromosome (Grinsted and Bennett 1988*a*), plasmids are thought to code for properties that are supplementary and nonessential for the host (Ohman 1988; Pelczar et al. 1993). Some of the properties associated with plasmids include the synthesis of bacteriocins, conjugation, dissimilation or degradation of various compounds, nitrogen fixation, utilization of sugars, resistance to various compounds including heavy metals and antibiotics, and the production of toxins (Pelczar et al. 1993; Prescott et al. 1990; Harwood 1993; Hardy 1981).

1.3.1. Plasmids of pseudomonads

The genus *Pseudomonas* contains a number of different plasmids, of varying sizes and encoding diverse properties. Amongst pseudomonads, plasmids range in size from 5.5 to 300 kbp (Grinsted and Bennett 1988*b*;

Hatfield 1990).

Many species of *Pseudomonas* are known to possess degradative or dissimilation plasmids. The TOL and NAH plasmids, which encode toluene and naphthalene oxidation, respectively, are perhaps the most extensively researched degradative plasmids found in pseudomonads (Bestetti et al. 1984). However, a number of degradative plasmids have been found in pseudomonads that degrade diverse compounds, including styrene, malonate, paraquat, 2,4-dichlorophenoxyacetate, and dibenzofuran (Bestetti et al. 1984; Kim and Kim 1994; Salleh and Pemberton 1993; Bhat et al. 1994; Selifonov et al. 1991). Resistance plasmids, especially those found in medically important strains of *P. aeruginosa*, are now receiving more attention (Boronin 1992). Many of these resistance plasmids encode enzymes with the ability to degrade antibiotics, such as the beta-lactams (Minami et al. 1996). Other plasmids encode resistance to metals, such as mercury and potassium tellurite (Salleh and Pemberton 1993; Suzina et al. 1995). In addition, pseudomonad plasmids have also been found to encode for compounds involved in bacteria-plant interactions, such as the ethylene producing genes of *Pseudomonas syringae* and the syringolide producing genes found in a different strain of *P. syringae* (Nagahama et al. 1994; Murillo et al. 1994). Consequently, considering the diverse properties encoded by plasmids from pseudomonads, it is conceivable that the plasmids of *P. fluorescens* strains T24 and T25 encode for the extracellular proteases produced by these strains.

1.4. Determination of the plasmid location of the protease gene(s)

Stanisich (1988) identified three possible technical approaches for determining whether or not a property of a bacterium is plasmid mediated. One is the presence of extrachromosomal DNA, that can be transferred to a suitable

host. A second approach is the transfer of the plasmid to a suitable host by one of three methods: conjugation, transformation or transduction. In both of these approaches, if the host bacterium successfully obtains the extrachromosomal DNA and the property, then the property is of plasmid origin. The final approach is to determine whether or not the bacterium would lose the property if the plasmid was either lost spontaneously or with the use of curing agents. If the property was lost when the plasmid was cured, then it is possible that the plasmid encodes the property. Consequently, three different techniques were used to determine whether or not the protease genes of *P. fluorescens* strains T24 and T25 are located on the plasmid DNA: conjugation, transformation, and curing.

1.5. Defining conjugation

First discovered by the experiments of Joshua Lederberg and Edward Tatum in 1946 (Prescott et al. 1990), conjugation is a process of DNA transfer from one bacterium (the donor) to another (the recipient) that involves direct cell-to-cell contact (Pelczar et al. 1993; Willetts and Wilkins 1984). The recipient bacterium that acquires the donor's genetic information is then referred to as a transconjugant (Provence and Curtiss 1994). Ohman (1988) indicates that conjugation is usually considered to be a plasmid encoded process, in which the newly synthesized molecule of plasmid DNA is transferred to another cell. However, chromosomal DNA is occasionally transferred during conjugation (Harwood 1993).

1.5.1. Conjugation in pseudomonads

Amongst the pseudomonads, conjugation has been reported to occur in

strains of *Pseudomonas putida*, *Pseudomonas cepacia*, and *P. syringae* (Sanseverino et al. 1993; Sabate et al. 1994; Bjorklof et al. 1995), but the most widely reported instances of conjugation involve clinically significant *P. aeruginosa* strains that contain plasmid encoded antibiotic resistance (Hupkova et al. 1994; Babalova et al. 1995). More importantly, however, conjugation has been reported to occur in *P. fluorescens*, with *P. fluorescens* acting as the donor bacteria and *Escherichia coli* and other bacterial species as the recipient(s) (Kim and Kim 1994; Richaume et al. 1992).

1.5.2. Conjugation experiments with *P. fluorescens*

To determine whether or not the protease genes of *P. fluorescens* strains T24 and T25 are located on the plasmid DNA, conjugation experiments were conducted in an attempt to transfer the plasmids to *E. coli* strains.

The experiment is based on the assumption that the plasmids present in the *P. fluorescens* strains are conjugative plasmids; naturally occurring plasmids that have the ability to mediate conjugation (Novick et al. 1976). In order for conjugation to occur, two separate, but equally important, sets of genes must be present. The transfer genes (*tra* genes) specify and /or control the synthesis of appendages in gram negative bacteria, termed donor pili. Pili are required for conjugative plasmids to allow the donor cells to make contact with the recipient cells, and also, to control substances that would otherwise minimize the occurrence of donor-recipient matings (Provence and Curtiss 1994). The second set of genes, the basis of mobility locus (*bom* locus), contains the site denoted the origin of transfer (*ori T*), the specific site where the transfer of DNA is initiated (Willetts and Wilkins 1984).

1.6. Transformation

First described by Griffith in 1928, before it was known that DNA was the genetic material, transformation is the process in which exogenous DNA is taken up by a bacterium, from its surrounding medium, and incorporated into the bacterial cell's genome (Chassy et al. 1988; Dale 1989; Saunders and Saunders 1988). For transformation to occur, the bacterial cell must have the ability to bind the exogenous DNA to the cell wall and transport it from the medium into the cell: that is, the cell must be competent (Mazodier and Davies 1991; Provence and Curtiss 1994). However, competence does not arise naturally in most genera of bacteria (Stanisich 1988).

According to Lorenz and Wackernagel (1994), there are a number of methods available to artificially induce competence in bacteria. These methods include treatment of cells with chelating agents such as EDTA; protoplast or spheroplast formation followed by fusion with DNA, the bombardment of cells with small particles, which can transport DNA into the cell, freezing and thawing of cells, treatment of cells with solutions of calcium chloride ($CaCl_2$) or chlorides of other elements (Mg, Ba, Rb, Sr), and electroporation (the exposure of cells to electric fields).

In this experiment, two different techniques were employed to artificially induce competence in *E. coli*: calcium chloride and electroporation. Calcium chloride induced competence is the most frequently used chloride in artificial transformation procedures in *E. coli* (Provence and Curtiss 1994). The $CaCl_2$ is said to render cell membranes more permeable to DNA: that is, the Ca^{2+} ion promotes DNA binding by neutralizing the negative charges of the cell membrane (Prescott et al. 1990; Saunders and Saunders 1988). The Ca^{2+} ion also causes the lipopolysaccharide and protein components of the outer membrane of gram negative bacteria to undergo rearrangements, that might help in the binding and/or transport of DNA molecules into the cell during

transformation (Saunders and Saunders 1988).

Electroporation is a relatively new and powerful technique that involves the application of a brief, high-voltage pulse to bacterial cells. The electrical pulse causes transient membrane distortions or small localized pores in the cell membrane, through which exogenous DNA can enter the bacterial cell (Provence and Curtiss 1994; Haynes and Britz 1990; Fiedler and Wirth 1988).

Transformation with the artificially induced competent *E. coli* cells was conducted using the whole plasmids of *P. fluorescens* strains T24 and T25, in an attempt to prove that the protease genes of these strains are located on the plasmid DNA. In addition, the efficiency of transformation for the two methods of artificially inducing competence will be compared.

1.7. Curing

The process of curing bacterial cultures of plasmids was first reported more than 35 years ago, when Hirota was able to artificially eliminate the F factor from *E. coli* (Crosa et al. 1994). Curing is the process by which a plasmid is eliminated from a culture as a whole, and does not refer to the physical removal of the plasmid from an individual cell (Dale 1989). Curing, whether it occurs spontaneously or is induced by treatments, is the result of an interference of the replication of the plasmid (Prescott et al. 1990). Consequently, as the bacterial cell divides copies of the plasmid DNA are not passed on to the daughter cells, and over several generations the plasmid becomes eliminated from the entire bacterial population (Dale 1989; Prescott et al. 1990).

Various agents have been used in curing experiments, including chemical agents (acridine orange, ethidium bromide, sodium dodecyl sulfate), physical agents (high temperature, low temperature, UV light), and recently, electroporation (Crosa et al. 1994; Dale 1989). The choice of curing agent and

the appropriate conditions varies widely among bacteria (Stanisich 1988).

In this experiment, the physical treatment of elevated temperature was employed. Heat treatment may cure bacteria of their plasmids if they show temperature-sensitive replication of the plasmid (Wells 1994). The use of elevated temperatures has been successfully employed to cure a number of bacteria of their plasmids, including, *Staphylococcus* (May et al. 1964), *Proteus* (Terawaki et al. 1967), *Pseudomonas facilis* (Pootjes 1977), *Rhizobium* (Xu and Murooka 1995), and *Rhodococcus equi* (Delapenamoctezuma and Prescott 1995).

1.8. Cloning

Hatfield (1990) produced a restriction endonuclease map of the plasmid pT20 from *P. fluorescens* strain T20, using single and double restriction endonuclease digests with seven enzymes *Sal* I, *Xba* I, *Hin*d III, *Pst* I, *Eco*R I, *Bam*H I, and *Sac* I. Hatfield (1990) was able to localize the protease gene from this strain to a 3.3 kbp *Sal* I fragment which was then inserted into pUC 12 to form a construct called pUT 8. However, the various pseudomonad strains produce differing amounts of extracellular protease and possibly have somewhat different protease gene(s). Therefore, the restriction endonuclease maps of the plasmids pT24 and pT25, from *P. fluorescens* strains T24 and T25, respectively, was determined and compared to that of pT20 from the *P. fluorescens* strain T20. In addition, the pUT 8 construct was non-radioactively labeled with digoxigenin and used as a probe to determine if and where the protease gene(s) are located on the pT24 and pT25 plasmids. The protease genes from the pT24 and pT25 plasmids were then inserted into the cloning vector pUC 18, which was used to transform *E. coli*.

1.9. Objectives

The objectives of this study are as follows:(1) to determine the genetic location of the protease genes in *P. fluorescens*, strains T24 and T25, (2) to construct a partial restriction endonuclease digestion map of the plasmids pT24 and pT25, and (3) to clone the protease genes from *P. fluorescens*, strains T24 and T25.

Chapter 2
Materials and Methods

2.1. Chemical reagents

The following chemicals were purchased from Sigma Chemical Company: sodium chloride, lactose, glucose, potassium chloride, magnesium chloride, magnesium sulfate heptahydrate, sodium hydroxide, ampicillin, sodium citrate dihydrate, maleic acid, Trizma (Tris), N-lauroylsacrosine, sodium dodecyl sulfate, m-cresol, β-mercaptoethanol, 8-hydroxyquinoline, hexadecyltrimethyl ammonium bromide, ethylenediamine tetraacetic acid, potassium acetate, calcium chloride, boric acid, piperazine-N,N'-bis(2-ethanesulfonic acid), lithium chloride, ammonium acetate, ethidium bromide, bromophenol blue, phenol, and sodium hydrogen carbonate.

Reagents purchased from Fisher Scientific Company include: trypticase soy agar, trypticase soy broth, oxidative-fermentative medium, motility medium, phenol red broth base, tryptone, yeast extract, glacial acetic acid, glycerol, bacteriological agar, isopropanol, isoamyl alcohol, chloroform, and ethanol.

2.2. Plasmid DNA

The pUC 18 plasmid was purchased from Boehringer Mannheim, while the pUT 8 construct was obtained from Hatfield (1990).

2.3. Bacterial strains

Pseudomonas fluorescens strains T24 and T25 were maintained on the appropriate selective media, trypticase soy agar (TSA) plates with 2% milk powder (MP).

Escherichia coli strains JM83, DH1 and DH5α were maintained on Luria Bertani (LB) agar plates (Davis et al. 1986).

2.3.1. Species confirmation tests

A battery of tests were conducted on the bacterial strains prior to experimentation. These tests included the gram stain, protease production, oxidation-fermentation test, acid and gas production in phenol red with glucose or lactose, motility test, catalase test, oxidase test, pigment production, and growth at 4°C versus 41°C.

2.4. Detection of protease negative colonies

P. fluorescens strains T24 and T25 each produce an extracellular protease which degrades the milk powder of the TSA + 2% MP plates in the vicinity of the colony, producing a distinct zone of lysis or halo around the colony. The zone of lysis is absent in protease negative colonies.

2.5. Bacterial growth media

For serial dilutions, 9 mL sterile, physiological saline blanks (0.85% NaCl) were prepared in standard culture tubes.

Trypticase soy broth (TSB) and Luria Bertani medium (LB; Davis et al. 1986) were used for the growth of the bacterial broth cultures.

Trypticase soy agar (TSA) plates with 2% MP were used for spread plating and screening of proteolysis.

For species confirmation, the following media and reagents were employed: oxidation-fermentation medium (OF); motility medium; phenol red broth base and phenol red glucose and lactose broth base with Durham tubes; catalase reagent; oxidase reagent.

2.6. Conjugation experiments

2.6.1. Broth mating protocols

Two broth mating protocols were used. The mating protocols (broth plate and filter) employed *P. fluorescens* strains T24 and T25 as the donor strains and the *E. coli* strains JM83, DH1 and DH5α as the recipient strains. The bacterial cultures were grown overnight (o/n), with shaking, in 10 mL of TSB, at 25°C for the *P. fluorescens* strains and at 37°C for the *E. coli* strains, to an optical density (600 nm) of approximately 1.0, unless stated otherwise.

In addition, to determine the initial number of colonies per mL of TSB, serial dilutions of the o/n *P. fluorescens* cultures were made, using saline blanks. One hundred microliter aliquots were spread plated, in duplicate, onto TSA + 2% MP plates, incubated for 24-48 hr at 25°C, and the number of colonies counted and used to determine the plate count.

Protocol 1

This protocol is a modification of the broth mating protocol of Williams and Murray (1974). A 0.2 mL aliquot of the o/n donor strain was mixed with a 1.0 mL aliquot of the o/n recipient strain and incubated for 0.5 hr at 25°C. The

mixture was then centrifuged at 13 500 rpm (Eppendorf Centrifuge 5414, Brinkmann) for 2 min. The pellet was resuspended in 1.5 mL of TSB , a 1.0 mL aliquot was removed and serially diluted in saline. From each of the 10^0 and 10^{-1} dilutions, 0.1 mL aliquots were plated onto ten TSA + 2%MP plates, incubated at 41°C and observed for protease positive transconjugant colonies (*P. fluorescens* is unable to grow at 41°C).

Modifications of the basic protocol were also performed. The volumes of the mixtures of the bacterial strains were modified; a 1.0 mL aliquot of donor culture and 4.5 mL of recipient culture were mixed with 4.5 mL of TSB. The time of incubation of the bacterial mixture was varied from 0.5 to 1.5 hrs, and finally, the temperature of incubation of the bacterial mixture was also modified to 37°C.

Protocol 2

The second broth mating protocol is a modification of the protocol of Ohman (1988). One loopful of culture was placed in 5 mL of LB and incubated o/n at 25°C and 37°C for the *P. fluorescens* and *E. coli* strains, respectively. The experimental 20 mL culture tube contained 2.0 mL LB, 0.2 mL of the donor culture and 0.2 mL of the recipient culture. Two controls were also used. Control A contained 2.0 mL of LB and 0.2 mL of donor culture. Control B contained 2.0 mL of LB and 0.2 mL of recipient culture. Both the experimental and control tubes were incubated at 37°C, with shaking at 100 rpm, for 2 hrs. A 100 µL aliquot was then diluted in a 900 µL saline blank to 1:10 (10^{-1}), and ten 100 µL aliquots of the 10^{-1} dilution were then spread plated onto separate TSA + 2% MP plates, which were incubated for 24-48 hrs at 41 °C. The colonies were observed for the presence of protease positive transconjugant colonies.

2.6.2. Plate mating

The protocol employed was a modification of the protocol given by

Willetts (1988). From the o/n cultures, 0.5 mL of both the donor and recipient bacteria were mixed, and a 0.3 mL aliquot was removed and spread plated onto TSA + 2% MP plates that were incubated o/n at 25°C. A 2.0 mL aliquot of TSB was then added to the plates to resuspend the cells, which were subsequently transferred to a 10 mL culture tube. The recovery procedure was repeated for a second time, and a 0.1 mL aliquot of this culture solution was then serially diluted in 9 mL saline blanks. From the 10^{-6} to the 10^{-8} dilutions, 100 µL aliquots were plated onto TSA + 2% MP plates (10 replica plates per dilution) and incubated at 41 °C for 48 hrs, at which time the plates were checked for protease positive transconjugants.

2.6.3. Filter mating

A modification of the protocol described by Willetts (1988) was employed. A 1.0 mL aliquot of each of the o/n bacterial cultures was placed in an eppendorf tube and centrifuged at 13, 500 rpm for 10 min at RT. The supernatants were discarded, and the pellets resuspended in 100 µL of TSB. A 0.45 µm pore size membrane filter (2.5 cm diameter, HA filter; Millipore) was aseptically placed onto a TSA + 2% MP plate, and 100 µL aliquots of the donor and recipient culture were then placed on the filter and incubated for 24 hrs at 37°C. The filter was then aseptically placed in 2.0 mL of TSB and the bacteria were resuspended. A 1.0 mL aliquot of the bacterial suspension was then serially diluted in 9 mL saline blanks and 100 µL aliquots of the 10^{-5} to the 10^{-8} dilutions were plated, in duplicate, onto TSA + 2% MP plates, then incubated for 24-48 hrs at 41°C. The plates were observed for protease positive transconjugant colonies.

2.6.4. Calculation of the rate (%) of conjugation

The rate of conjugation was calculated by dividing the number of transconjugants by the initial number of donor colonies added to the conjugation mixture.

2.7. Transformation using calcium chloride

2.7.1. Preparation of competent cells

The protocol used was a modification of that given by Ausubel et al. (1995). A loopful of 24-48 hr *E.coli* culture was inoculated into 50 mL of LB and incubated o/n, with shaking (150 rpm), at 37°C. From the o/n culture, 4 mL was inoculated into 400 mL of LB medium in a 2-liter flask, and the culture was grown at 37°C to an OD_{590} of approximately 0.375. The culture was then aliquoted into eight 50 mL prechilled, sterile polypropylene tubes, and incubated on ice for approximately 10 min. The culture was then centrifuged for 7 min at 1600 x *g*, 4°C. The supernatant was carefully discarded and each pellet resuspended in 10 mL ice-cold $CaCl_2$ solution (60 mM $CaCl_2$; 15% glycerol; 10 mM PIPES, pH 7.0). The cells were centrifuged for 5 min at 1100 x *g*, 4°C. The supernatant was discarded and each pellet was resuspended in 10 mL of $CaCl_2$ solution and incubated on ice for 30 min. The cells were centrifuged again for 5 min at 1100 x *g*, 4°C, the supernatant discarded, and the pellet resuspended in 2 mL of ice-cold $CaCl_2$ solution. The cells were then dispensed into prechilled, sterile polypropylene tubes (250 µL) and immediately frozen at -70°C. The competency of the cells was assessed using pUC 18 plasmid DNA and LB agar plates containing 0.1M isopropyl-β-D-thiogalactopyranoside (IPTG), 2% 5-bromo-4-chloro-3-indolyl-β-D galactoside (X-gal) and 50 µg/mL ampicillin (Amp)

(see appendix1).

2.7.2. Transformation of competent cells

An aliquot of approximately 10 ng of plasmid DNA in a final volume of 10-25 µL was placed into a prechilled, sterile 15 mL round-bottomed test-tube and placed on ice. The competent cells were rapidly thawed by warming between the hands and 100-150 µL was immediately added to the test tube containing the DNA. The tube was gently swirled to mix the contents and incubated on ice for approximately 10 min. The cells were then heat shocked in a water bath at 42°C for 2-3 min or 37°C for 5 min. To the cells, 1 mL of LB (prewarmed) was added and the cells were incubated with shaking at 150 rpm for 1 hr at 37°C. The cells were then serially diluted in saline to 10^{-6}, and 100 µL aliquots of each dilution were plated, in duplicate, onto TSA + 2% MP plates and incubated o/n at 37°C. The dilution containing between 30-300 colonies was then used to plate additional 100 µL aliquots (10-20) onto TSA + 2% MP plates, which were again incubated at 37°C. The plates were observed for protease positive transformant colonies; that is, colonies surrounded by a distinct zone of lysis. For each plasmid DNA / *E. coli* combination, there was a control sample, lacking the DNA, that was subjected to the identical conditions.

2.8. Transformation using electroporation

2.8.1. Preparation of cells

The bacterial cells were prepared according to the protocol found in the instruction manual for the electroporation apparatus. One loopful of culture was placed in 10 mL of LB and incubated o/n at 37°C or 25°C, for *E. coli* and *P.*

fluorescens, respectively. The o/n culture was then placed in 1 L of LB and incubated at the appropriate temperature, with shaking (~150 rpm), to an OD_{550} of 0.6. The cells were then transferred to centrifuge bottles and chilled on ice for approximately 20 min, before they were harvested for 10 min, 10 000 x *g*, at 4°C. The supernatant was discarded and the pellets were each washed with 10 mL of sterile, ice-cold, distilled water, before being centrifuged again. The pellets were then washed twice with a 10% solution of ice-cold glycerol, with centrifugation after each rinsing. The pellets were then resuspended in a total volume of approximately 2 mL of the 10% glycerol solution and frozen in 100 µL aliquots.

2.8.2. Electroporation of cells

Electrode cuvettes, 0.1 cm size with a maximum volume of 80 µL, and the Porator electroporation apparatus (Invitrogen Corporation) were used in the electroporation experiments. The volume of DNA to be used for the electroporation experiment was first determined, using 5% volume or less of the volume of the competent cells. The DNA concentration used was approximately 1 µg. Generally, 1-2 µL of DNA and 40 µL of competent cells were added to an eppendorf tube and placed on ice, at which time the cuvettes were also placed on ice. The electroporation settings used were those suggested by the manufacturer. For the electroporation apparatus, a capacitance of 50 microfarads (µF); a load resistance of 200 ohms (Ω);a pulse width of approximately 5-8 msec; and voltages of 1000 and/or 1500 V were employed, resulting in field strengths of 10 and 15 kV/cm. The electroporation apparatus was tested at each voltage prior to use, with a blank cuvette chamber to ensure that the apparatus was working properly. The rocker switch was placed in the charge position and the unit was allowed to develop a full charge (~ 30 sec). The DNA/cell solution was then transferred to the electrode cuvette. The

cuvette was gently tapped to remove any air bubbles and to bring the solution down to the bottom, and the cap was placed on the cuvette. The outside surfaces of the cuvette were wiped dry with a kimwipe, before inserting the cuvette into the cuvette chamber, notch facing forward, and the chamber lid was closed. The rocker switch was then switched to the discharge position and left there. The pulse light indicated whether or not the charge had been delivered to the cuvette chamber, and when the unit had been fully discharged. The cuvette was immediately removed from the chamber and approximately 2 mL of SOC (2% tryptone; 0.5% yeast; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4 \cdot 7H_2O$; 20 mM glucose) was added to the cells before transfer to a sterile 15 mL Falcon tube. The cells were incubated for 1 hr at the appropriate temperature, at which time they were diluted to 10^{-4} using (9 mL blanks of) physiological saline. Duplicate samples of 100 µL were plated for each dilution, and the plates (LB plates with IPTG, Xgal, and Amp for *E. coli* and TSA + 2% MP plates for *P. fluorescens*) were incubated o/n at the appropriate temperature. The plates were observed to determine which dilution contained 30 - 300 colonies. From the appropriate dilution 10-20 additional plates were spread plated. The plates were observed for protease positive transformant colonies. For each experiment, there was a control containing only the bacterial cells, that were not subjected to any voltage, but which were grown in SOC media, serially diluted, and plated onto TSA + 2% MP plates, as were the experimental samples.

2.9. Heat treatment curing experiment

One loopful of an overnight protease positive streak plate culture of *P. fluorescens* (strains T24 and T25) was inoculated into 5 mL of LB and incubated

o/n at 25°C. A 0.2 mL aliquot of this culture was added to 2 mL of LB and incubated for 2 hrs at 37°C, on a shaker (150 rpm). Following temperature treatment, the broth culture was diluted 1:10 (0.1 mL in 0.9 mL of saline). Ten 100 µL aliquots of the dilution were plated onto TSA + 2% MP plates and incubated for 24-48 hrs at 37°C, at which time the colonies were observed for protease activity.

2.10. Large scale plasmid DNA extraction

A modification of the Maniatis et al. (1982) large-scale isolation of plasmid DNA by alkaline lysis was employed. The *Pseudomonas* cultures were not amplified. One liter of LB was inoculated with one loopful of o/n culture and incubated for 12-16 hrs at 25°C in a shaker (150 rpm). The cells were harvested at 8 000 x *g*, for 10 min., at 4°C. The supernatant was discarded and the pellet was resuspended in physiological saline (60 mL) and harvested as previously. The supernatant was discarded and the pellet resuspended in 20 mL of solution I (50 mM glucose; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 7.5), and incubated at RT for 10 min. A 40 mL volume of solution II (200 mM NaOH; 1% SDS, pH 12.45) was added and the mixture was gently inverted before incubation on ice (45 min). A 30 mL volume of solution III (3 M KOAc, pH 4.8) was then added and the mixture incubated on ice for 10 min, followed by centrifugation (14 500 x *g*, 45 min, 4°C).

The supernatant was extracted with an equal volume of phenol mixture (1.6 M phenol; 16 mM Tris-HCl, pH 8.0; 40 mM NaOH) . To the aqueous layer, an equal volume of 7.5 M ammonium acetate was added, the mixture incubated on ice for 10 min (20-30 min if precipitation occurs) and subjected to centrifugation (14 500 x *g*, 10 min, RT). To the supernatant a 0.6 volume of isopropanol was added, incubated at RT for a minimum of 15 min, followed by

centrifugation (14 500 x *g*, 45 min, 12°C). The pellet was washed with 70% ethanol, dried under a vacuum and redissolved in 1 mL of 1/10 Tris-HCl EDTA buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0).

2.11. Determination of concentration of DNA

The concentration of DNA in a sample was determined according to the protocol of Davis et al. (1986). To 995 µL of distilled water, 5 µL of the DNA sample was added, and the two were mixed. The sample was then placed in the cuvette and the absorbance at 260 nm was taken with a Spectronic 601 spectrophotemeter. The concentration of DNA, in µg/µL, is 10x the OD_{260} reading.

2.12. Agarose gel electrophoresis

Horizontal agarose gel electrophoresis was carried out in 0.8% agarose (Fischer Biotech) in 1x Tris-Borate-EDTA buffer (89 mM Tris; 89 mM boric acid; 2 mM EDTA, pH 8.0), containing 6 µL/mL ethidium bromide (EtBr). Samples were combined with 10 µL of Loading buffer (0.25% bromophenol blue; 40% sucrose; 10% glycerol), loaded into the wells and the gels were subjected to electrophoresis at 80-100 volts, for 2-3 hrs, at RT. The gels were photographed with a red filter using a polaroid camera (Polaroid MP-4 Land camera; Fotodyne Inc.) and Kodak 660 film.

2.13. Restriction endonuclease digestion

Restriction endonuclease digestion of plasmid DNA, from *P. fluorescens*

strains T24 and T25, was carried out with the restriction endonucleases *Sal* I, *Xba* I, *Hind* III, *Pst* I, *Eco*R I, *Bam*H I, and *Sac* I (Pharmacia Biotech Inc.). All reactions were carried out in 20 µL volumes. The single digests for the first four restriction endonucleases consisted of 10 µL of plasmid DNA (1-2 µg), 1.0 µL of restriction endonuclease (1-2 units), 2.0 µL of buffer and 7.0 µL of sterile, distilled water. The single digests for the latter three enzymes differed from that of the first four in the volumes of buffer and water, 4.0 µL and 5.0 µL, respectively. The double digests consisted of 10 µL of plasmid DNA, 0.75 µL of each restriction endonuclease, 2.0 µL of buffer and 6.5 µL of sterile, distilled water. All reactions were incubated for 6-8 hrs at 37°C, and stopped by heating at 85°C.

2.14. Determination of molecular weight of fragments

The *Hind* III restriction endonuclease digests of bacteriophage lambda (λ) DNA (Pharmacia Biotech. Inc) were used to prepare standard curves. The distances migrated by the *Hind* III digested λ DNA fragments were plotted against the logarithm of their size in kilobase pairs (kbp). The standard curves were then used to determine the molecular weight of the restriction digested plasmid DNA from the *P. fluorescens* strains T24 and T25 (pT24 and pT25), using their distances of migration.

The restriction endonuclease digest map was generated manually from both the single and double digests of the pT24 and pT25 plasmids.

2.15. Southern transfer

Following restriction endonuclease digestion, and agarose gel

electrophoresis, the pDNA fragments were immobilized onto positively charged nylon membranes (Boehringer Mannheim) according to the protocol of Ausubel et al. (1995). The gel was washed with distilled water and then placed in 10 gel volumes of 0.25 M HCl for up to 30 min (when the bromophenol blue dye has turned yellow wait an additional 10 min), at RT, with shaking, to partially depurinate the DNA. The gel was rinsed with distilled water, and then placed in 10 gel volumes of 0.4 M NaOH for 20 min, at RT, with shaking, to denature the DNA. The DNA fragments were then transferred to a positively charged nylon membrane in 0.4 M NaOH, using an upward transfer protocol (see appendix 2).

2.16. Hybridization and post-hybridization treatment

Following Southern transfer of the pDNA fragments onto the positively charged, nylon membrane, the membrane was incubated for 30 min at 68°C in prewarmed hybridization buffer (5x SSC, 0.1%N-lauroylsacrosine, 0.02% SDS, 1% Blocking Reagent; 20 mL/ 100 cm^2 membrane). The DIG-labeled DNA probe (5-25 ng/mL) was denatured by boiling in a water bath for 5 min followed by rapid cooling on ice. The probe was then added to prewarmed hybridization buffer (2.5 mL/ 100 cm^2 membrane) and mixed well. The original hybridization solution was then removed from the membrane and replaced by the probe/ hybridization mixture and incubated at 68°C for a minimum of 6 hrs. The membrane was removed from the probe/ hybridization mixture and washed twice, for 5 min, in 2x SSC, 0.1% SDS solution at RT (50 mL/ 100 cm^2 membrane) and then twice, for 15 min, in 0.1x SSC, 0.1% SDS solution at 68°C. The membrane was then air dried before immunological detection.

2.17. Digoxigenin DNA labeling and detection

The protocols employed were those provided with the digoxigenin (DIG) DNA labeling and detection kit (Boehringer Mannheim). Solution preparation is described in appendix 1.

2.17.1. DIG DNA labeling

The template DNA (0.5-3 µg of pUT 8) was diluted to a total volume of 15 µL, denatured by heating in a boiling water bath for 10 min, and quickly chilled on ice. To the denatured DNA, 2 µL of hexanucleotide mix, 2 µL of dNTP, and 1 µL of Klenow enzyme were added, the mixture briefly centrifuged, and incubated for approximately 20 hrs at 37°C. The reaction was stopped by the addition of 2 µL of 0.2 M EDTA (pH 8.0) and the labeled DNA was precipitated by the addition of 25 µL of 4 M lithium chloride (LiCl) and 75 µL of prechilled absolute ethanol. The mixture was incubated at -20°C for 2 hrs, followed by centrifugation for 15 min. The pellet was washed with 50 µL of 70% ethanol, briefly dried under a vacuum, dissolved in 50 µL of TE buffer (pH 8.0) and stored at -70°C.

2.17.2. Immunological detection

The membrane was rinsed in maleic acid buffer for 1-5 min, followed by incubation in 100 mL blocking solution for 30 min, at RT. The anti-DIG-AP conjugate was diluted 1:5000, and the membrane was incubated in the antibody solution for 30 min. The membrane was washed twice with 100 mL maleic acid buffer, and equilibrated for 5 min in 20 mL detection buffer. The membrane was incubated for a minimum of 5 min in 10 mL of colour solution (containing X-phosphate and NBT), in the dark. To stop the reaction when desired spot/band intensities were achieved, the membrane was washed in 50 mL of distilled water for 5 min.

2.18. Electroelution of DNA from agarose gel

Electroelution of the DNA from the agarose gel was according to the protocol of Davis et al. (1986).

2.18.1. Preparation of dialysis tubing

Dialysis tubing of 13 mm diameter was cut into 25 cm lengths and placed in a solution of 2% $NaHCO_3$, 1 mM EDTA. The solution was heated until boiling and boiled for 1 min. The solution was allowed to cool and the tubing was rinsed three times in distilled water, twice in absolute ethanol, and twice in 1 mM EDTA (pH 8.0), before storage at 4°C in 1 mM EDTA (pH 8.0).

2.18.2. Electroelution

The DNA of interest was run on a 0.8% agarose gel. The DNA bands were visualized at 300 nm on a UV transilluminator (Fotodyne, Bio-Can Scientific), the desired DNA bands were cut from the gel using a scalpel, and the bands were placed in individual dialysis bags with one end clipped. A volume of 450 µL of 0.2x TBE buffer was added, the air squeezed from the bag and the second end of the bag clipped. Excess tubing was removed and the bag was checked for leaks. The dialysis bag(s) were placed in a horizontal agarose gel apparatus, perpendicular to the line between the electrodes, and the gel apparatus was then filled with 0.2x TBE, just sufficient to cover the bag. A 300 V voltage was applied for 1-3 hr, the polarity reversed and then run for an additional 1-2 min. The solution was carefully removed from the bag and placed in a 1.5 mL eppendorf tube. The dialysis bag was rinsed with an additional 450 µL of 0.2x TBE, and the two volumes were combined. The sample was

centrifuged for 15 sec to pellet any transferred gel pieces, and the supernatant was transferred in two 450 µL aliquots to two 1.5 mL eppendorf tubes. The DNA was then purified using the salt saturated phenol/chloroform extraction protocol below.

2.19. DNA extraction and precipitation

The electroeluted DNA samples were purified according to the protocol of Davis et al. (1986). To each DNA sample, 1 volume of salt saturated phenol (see appendix 1) and 1 volume of chloroform was added and the solution mixed by repeated gentle inversions. The samples were then centrifuged in a microcentrifuge for 1 min at 4°C. The upper aqueous layer was removed and transferred to a new 1.5 mL eppendorf tube. A 1/10 of 1 volume of 3 M sodium acetate (pH 7.0) was added followed by at least 2.5 volumes of 95% ethanol. The DNA solution was placed at - 20°C for 1-3 hours and then centrifuged for 10 min at 4°C. The supernatant was carefully removed, the pellet was washed with 300 µL or more of 80% ethanol, and again centrifuged for 2 min at 4°C. The supernatant was discarded and the pellet air dried before being suspended in TE (pH 8.0).

2.20. Bacterial genomic DNA extraction

Genomic DNA was extracted from bacteria according to the protocol of Ausubel et al. (1995). A 5 mL volume of TSB was inoculated with *P. fluorescens*, strains T24 and T25, respectively, and the cultures were grown o/n at 25°C. A 1.5 mL aliquot of the culture was centrifuged for 2 min at RT. The supernatant was discarded and the pellet was resuspended in 567 µL of TE

buffer by repeated pipetting. A 300 µL volume of 10% SDS and 3 µL of 20 mg/mL proteinase K (Boehringer Mannheim) was added to give a final concentration of 100 µg/ mL proteinase K in 0.5% SDS. The solution was mixed thoroughly and incubated for 1 hr at 37°C. A 100 µL volume of 5 M NaCl was added and the solution thoroughly mixed before the addition of 80 µL of CTAB/NaCl (10% CTAB in 0.7M NaCl) and incubation at 65°C for 10 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added, the solution thoroughly mixed, and centrifuged for 4-5 min at RT. The aqueous supernatant was removed, transferred to another eppendorf tube, and extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1; see appendix 1) before centrifugation for 5 min at RT(Davis et al. 1989). The supernatant was transferred to a fresh tube, 0.6 volume of isopropanol was added, and the tube was gently shaken back and forth until a stringy white DNA precipitate was visible. The DNA pellet was transferred to a new tube, washed with 70% ethanol, and centrifuged for 5 min at RT. The supernatant was carefully removed and the pellet dried under vacuum before resuspension in 100 µL of TE buffer (pH 8.0).

2.21. Subcloning of DNA fragments

The experiments were performed according to the protocol of Ausubel et al. (1995). The individual DNA components (pT24, pT25, genomic T24 DNA, genomic T25 DNA, and pUC 18) were cleaved with the appropriate restriction endonuclease (*Sal* I, *Pst* I or *Bam*H I) in a 20 µL reaction volume, for 8 hr at 37°C. The enzymes were then inactivated by heating 15 min at 85°C. To remove the 5' phosphates of the pUC 18 digests, 2 µL of 10 X calf intestinal phosphatase (CIP; Pharmacia Biotech Inc.) buffer and 1 unit (U) CIP were added to the reaction mixture and incubated 30 to 60 min at 37°C. The CIP was

inactivated by heating for 15 min at 85°C. The DNA fragments were purified via the ss-phenol purification protocol. The ligation reaction consisted of 9 µL of component DNAs (0.1 to 5 µg), 10 µL of 2 X T4 DNA ligase buffer, 1 µL of 10 mM ATP (Pharmacia Biotech Inc.), and 20 to 500 U (Weiss) of T4 DNA ligase (Gibco BRL). The reaction mixture was then incubated for up to 24 hr at 15°C. Following the incubation, 1 to 10 µL of the ligated products were introduced into *E. coli*, strain DH5α, via the $CaCl_2$ transformation protocol. Controls were also employed simultaneously, in which one of the DNA components were lacking from the ligation reaction mixture.

Chapter 3
Results

3.1. Conjugation experiments

Approximately 40 000 colonies were screened in the conjugation experiments, as indicated in Table 3.1. No protease-positive transconjugants were detected.

3.2. Transformation experiments

For the transformation experiments with $CaCl_2$, three strains of *E. coli*, JM83, DH1 and DH5α, and the plasmid DNA pT24 and pT25 were used. A total of 47 857 colonies were screened: no protease positive transformants were detected (Table 3.2).

For the transformation experiments with electroporation, two strains of *E. coli*, JM83 and DH5α, and the plasmid DNA pT24 and pT25 were used. A total of 178 027 colonies were screened: no protease positive transformants were detected (Table 3.3).

3.3. Heat treatment experiments

Following the heat treatment of *P. fluorescens* strain T24 no protease negative colonies were detected among 17,416 colonies screened (Table 3.4).

Heat treatment of *P. fluorescens* strain T25 did result in the production of protease negative colonies (Fig 3.1). As indicated in Table 3.5, of the 1671 colonies screened, 144 were initially protease negative. Upon reculturing, 66 of

Table 3.1. Conjugation experiments between *P. fluorescens*, strains T24 and T25, and *E. coli*, strains JM83, DH1 and DH5α.

Bacterial Combinations	Number of Colonies Screened	Number of Transconjugants
T24 + JM83	8729	0
T24 + DH1	6870	0
T24 + DH5α	5459	0
T25 + JM83	3948	0
T25 + DH1	3207	0
T25 + DH5α	8887	0

Table 3.2. Transformation experiment using $CaCl_2$ competent JM83, DH1 and DH5α strains of *E. coli* and the plasmids pT24 and pT25, isolated from *P. fluorescens* strains T24 and T25, respectively.

E. coli / plasmid DNA Combination	Number of Colonies Screened	Number of Transformants
JM83 / pT24	2 646	0
DH1 / pT24	3 650	0
JM83 / pT25	18 253	0
DH1 / pT25	16 089	0
DH5α / pT25	7 215	0

Table 3.3. Transformation experiments, using the technique of electroporation, involving the *E. coli* strains JM83 and DH5α, and the plasmids pT24 and pT25, isolated from *P. fluorescens* strains T24 and T25, respectively, using two different temperatures of incubation, 25°C and 37°C.

E. coli / Plasmid DNA Combinations	Number of Colonies Screened		Number of Transformants
	25°C	37°C	
JM83 / pT24 (1000V)[a]	5 797	4 982	0
JM83 / pT24 (1500V)	6967	15 597	0
JM83 / pT25 (1000V)	4 034	6 764	0
JM83 / pT25 (1500V)	19 996	8 601	0
DH5α / pT24 (1000V)	9 968	15 588	0
DH5α / pT24 (1500V)	5 357	10 292	0
DH5α / pT25 (1000V)	8 560	34 879	0
DH5α / pT25 (1500V)	4 961	15 677	0

[a] The electroporation experiments for each of the *E. coli* / plasmid DNA combinations were conducted at two different voltages, 1000 V and 1500 V.

the original 144 colonies remained viable and protease negative, 12 were protease positive, and the remaining 66 were not viable.

3.3.1. Screening protease negative colonies for plasmid DNA

Of the 66 protease negative colonies, three were selected for plasmid extraction. To confirm that a protease negative *P. fluorescens* colony had been cured of its plasmid DNA, a modification of the Maniatis et al. (1982) large-scale isolation of plasmid DNA by alkaline lysis was employed. A protease positive *P. fluorescens* colony was used as a positive control for the plasmid DNA extraction protocol, and the cultures were not amplified. The results of the extraction indicated that the three colonies were cured and did not contain the plasmid DNA that was present in the protease positive T25 colony. Figure 3.2 illustrates the absence of plasmid DNA in one of the three cured colonies.

3.3.2. Confirmation of the classification of the protease negative colonies

A series of tests were conducted on the protease negative *P. fluorescens* colonies. Biochemical tests included oxidase, catalase, indole, citrate, arginine and malonate utilization, and lactose, maltose and meso-erythritol fermentation. Growth at 4°C and 41°C, fluorescent pigment production, motility and gram staining were also tested. All of the tests indicate that the protease negative colonies screened were *P. fluorescens*.

3.3.3. Reintroduction of plasmid DNA into cured *P. fluorescens*, strain T25

Following electroporation no protease positive colonies were detected among the 3 744 colonies screened (Table 3.6).

Table 3.4. The effect of an elevated temperature of 37°C on curing *P. fluorescens* strain T24 of its plasmid.

Total Number of Colonies Screened	Total Number of Protease Negative Colonies
17 416	0

Table 3.5. Generation of protease negative colonies of *P. fluorescens* strain T25 at an elevated temperature of 37°C.

Initial Screening		Subcultured Protease Negative Colonies	
Protease + Colonies	Protease - Colonies	Protease + Colonies	Protease - Colonies
1527(91)[a]	144(8.6)	14(17.5)	66(82.5)

NOTE: Freshly grown *P. fluorescens* strain T25 was inoculated into LB and incubated at 37°C for 2 hrs. Serial dilutions of the culture were plated on TSA + 2%MP plates. Protease positive colonies showed clearing around the colonies, indicating hydrolysis of milk proteins.

[a] Values in parentheses represent percentages of colonies screened.

(+) Positive

(-) Negative

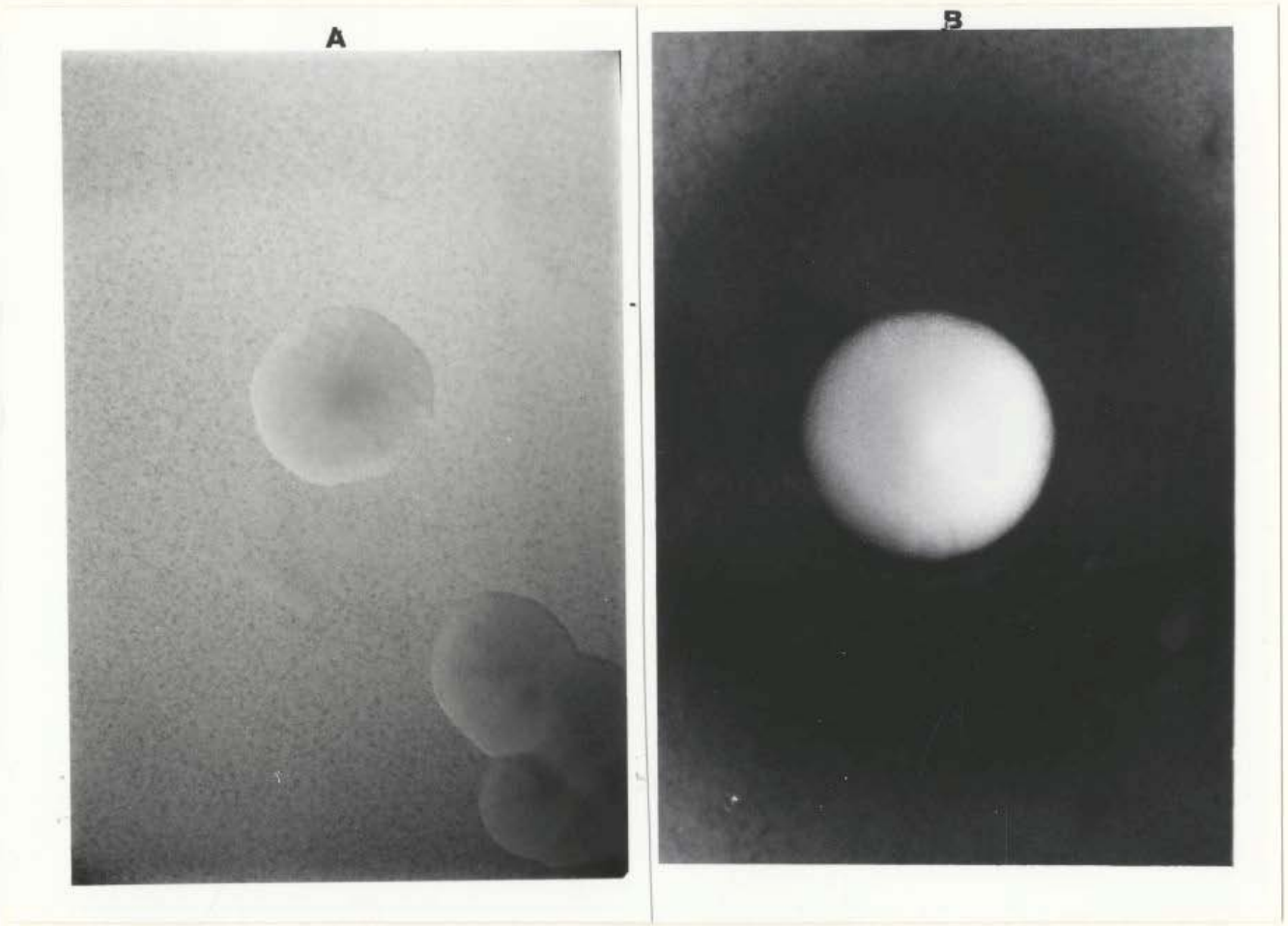

Figure 3.1. Colonies of *P. fluorescens* strain T25. (a) Heat cured protease negative colonies, lacking zone of lysis. (b) Protease positive colony showing the zone of lysis in the media surrounding the colony. Pictures were taken on a dark background.

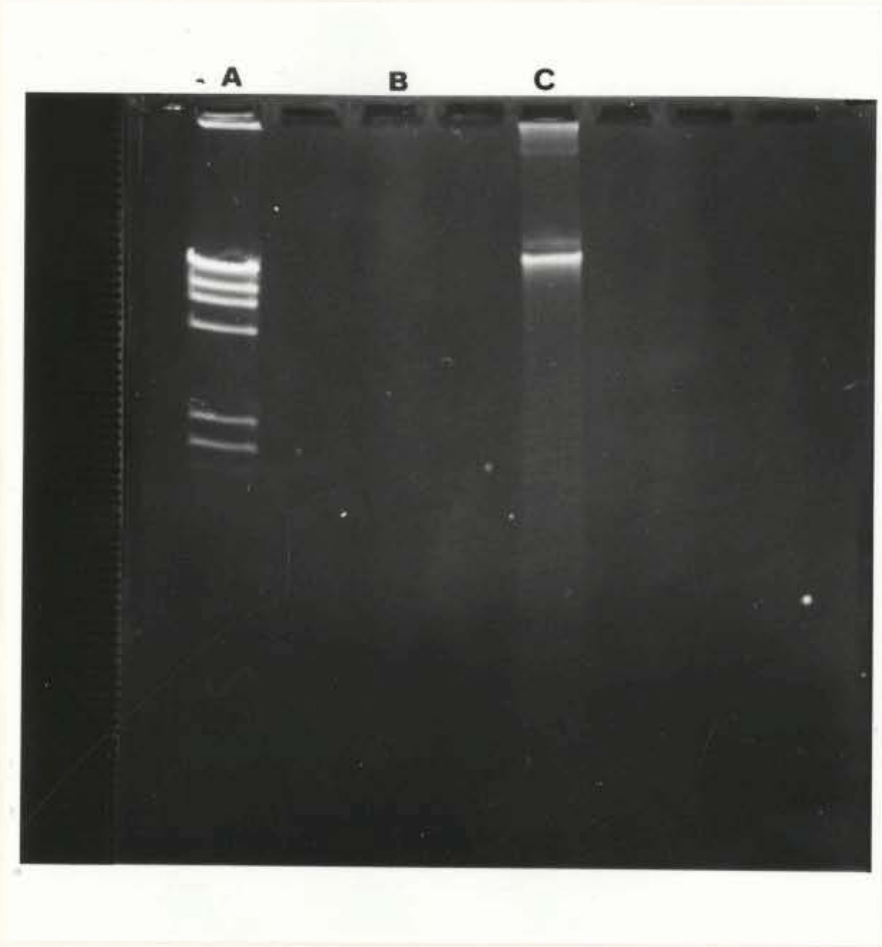

Figure 3.2. Agarose gel electrophoresis of *P. fluorescens* strain T25 plasmid DNA. Lane A contains a *Hind* III digested lambda DNA marker. Lane B indicates the absence of plasmid DNA from a cured, protease negative *P. fluorescens* colony. Lane C contains the plasmid DNA from a protease positive colony of *P. fluorescens* strain T25.

Table 3.6. *Transformation* of cured *P. fluorescens* strain T25 with plasmid DNA from untreated *P. fluorescens* strain T25 colonies, using electroporation.

Number of Colonies Screened	Number of Transformants
3744	0

3.4. Estimation of the molecular weights of the plasmids

The plasmids from *P. fluorescens* strains T24 and T25 appeared to have high molecular weights. To estimate the molecular weights of the plasmids pT24 and pT25, a series of single and double restriction endonuclease digests were carried out on each of the plasmids. The fragments generated from the digests (Figures 3.3-3.6 and 3.7-3.10) and the sizes of the various fragments (Tables 3.7-3.10 and 3.11-3.14) were all used to determine the molecular weights of the plasmids. The average sizes for the plasmids were calculated to be 36.38 kbp and 34.57 kbp, for the pT24 and pT25 plasmids, respectively.

3.5. Construction of the restriction endonuclease digest map of *P. fluorescens* strains T24 and T25

Restriction endonuclease digestion map for pT24 and pT25 were obtained using the information provided by both the single and double restriction endonuclease digests. The sizes of both the single and double restriction endonuclease digests fragments and the digest patterns are similar for both the pT24 and pT25 plasmids. The map (Fig 3.11) is based on the fragments derived from all seven of the restriction endonucleases used. On the basis of the enzymes used, pT24 and pT25 have the same restriction map. However, the pT24/pT25 restriction endonuclease digestion map is significantly different from that of pT20 (Fig. 3.12).

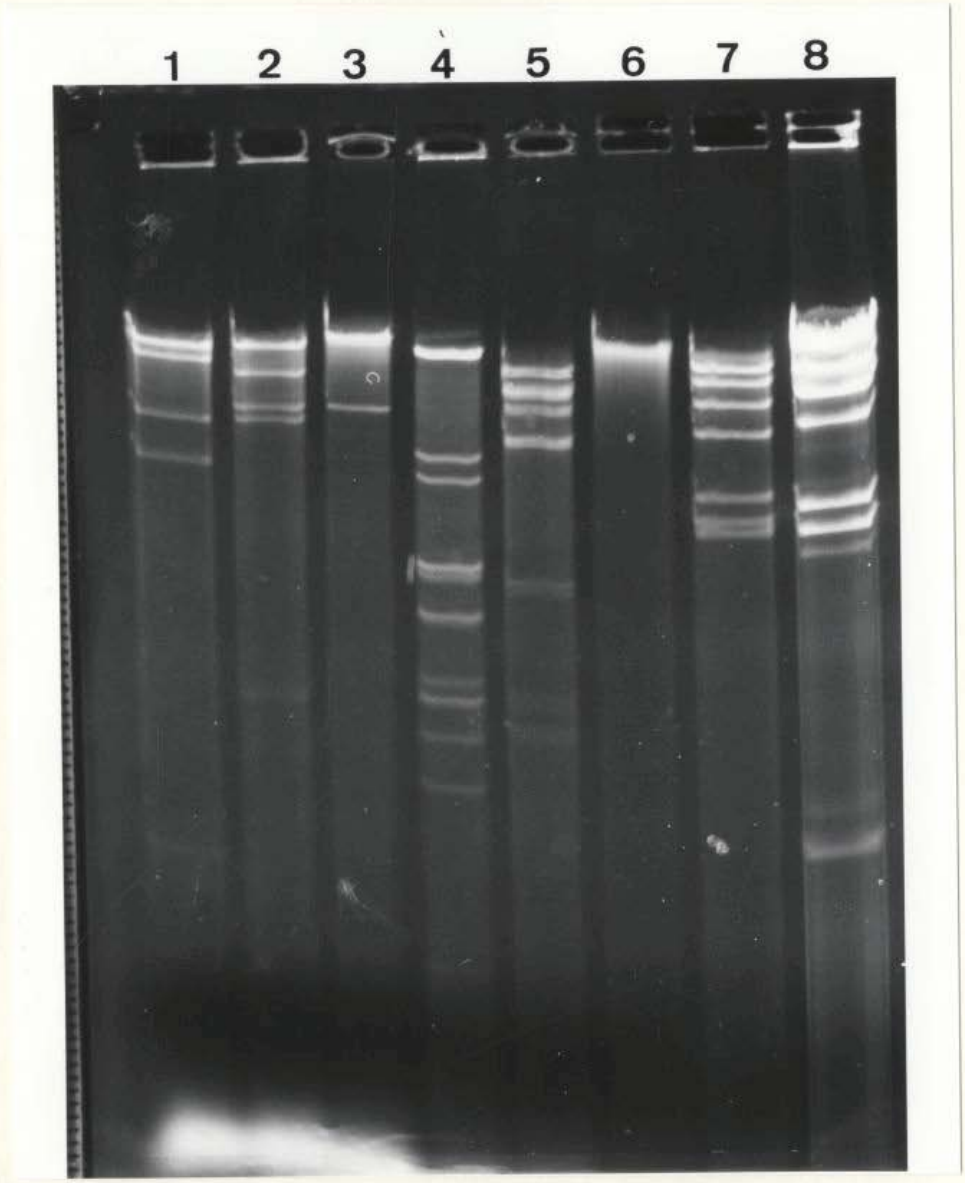

Figure 3.3. Restriction endonuclease single digest fragments of pT24. Lane 1 = *Bam*H I, lane 2 = *Eco*R I, lane 3 = *Sac* I, lane 4 = *Pst* I, lane 5 = *Sal* I, lane 6 = *Xba* I, lane 7 = *Hin*d III, and lane 8 = *Hin*d III digested λ DNA Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Table 3.7. Molecular weight of fragments derived from single restriction endonuclease digests of the pT24 plasmid.

Restriction Endonuclease	Number of Fragments	Fragment Size (kbp)	Total Size (kbp)
Hind III	7	11.0, 8.2, 5.0, 4.0, 2.35, 2.07, 1.72	34.34
Xba I	1	23.0 [a]*	23.0
Sal I	8	9.0, 7.2, 5.2, 3.75, 3.3,1.02, 0.99, 0.9	31.36
Pst I	11	23.0 [a], 3.4, 2.8, 1.72, 1.69, 1.3, 1.39, 1.08, 1.0, 0.86, 0.71	38.95
Sac I	2	28.0 [a], 5.6	33.6
*Eco*R I	5	20.0, 10.2, 6.1, 5.2, 1.0	42.5
*Bam*H I	5	20.0, 10.5, 5.6, 3.75, 0.6	40.45
Hind III digested λ DNA	7	23.0, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56	

[a] Estimation of the size of the single band and bands migrating at approximately the same distance as the 23.0 kbp of the *Hind* III digested λ DNA marker is not a very reliable measure of the actual size of the bands.

*This fragment was not used in the calculation of the plasmid size.

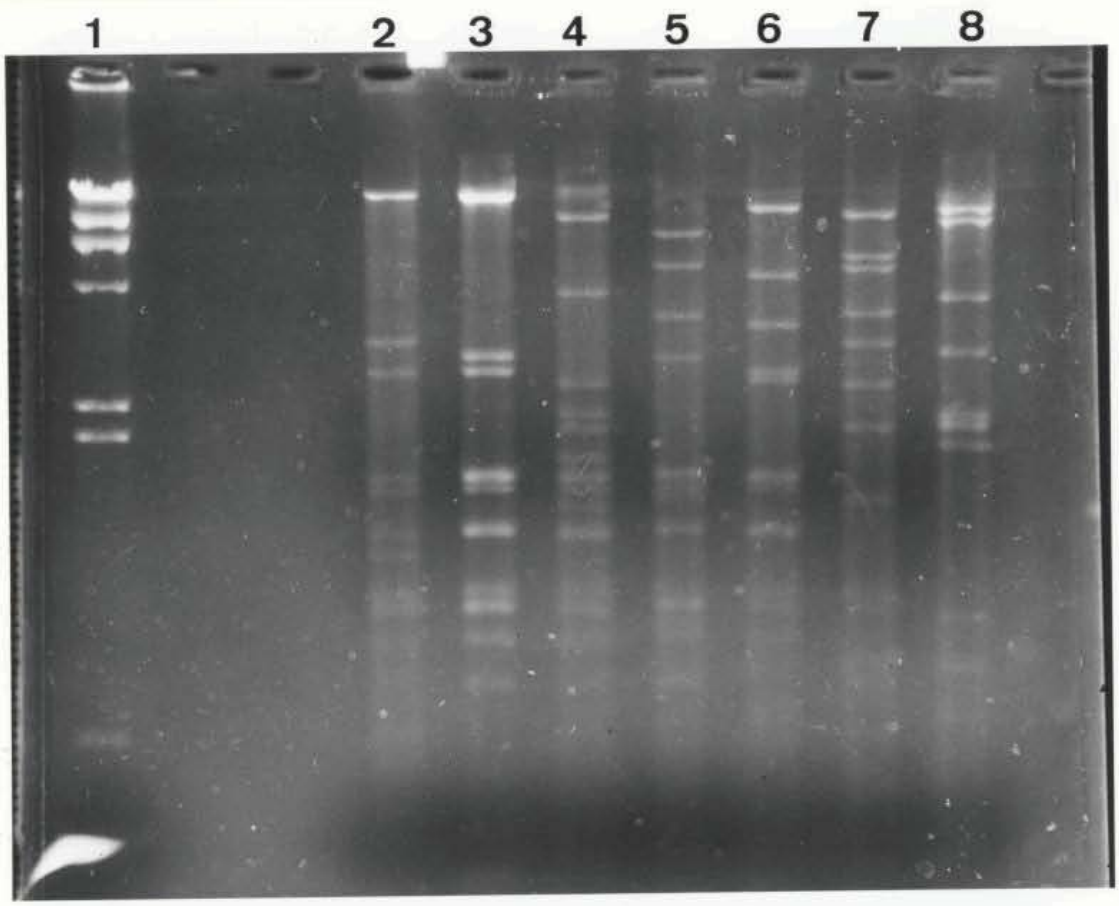

Figure 3.4. Restriction endonuclease double digest fragments of pT24. Lane 1 = *Hind* III digested λ DNA, lane 2 = *Pst* I / *Bam*H I, lane 3 = *Pst* I / *Xba* I, lane 4 = *Pst* I / *Hind* III, lane 5 = *Pst* I / *Sal* I, lane 6 = *Pst* I / *Eco*R I, lane 7 = *Hind* III / *Sal* I lane 8 = *Hind* III / *Eco*R I. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Table 3.8. Molecular weight of fragments derived from double restriction endonuclease digests of the pT24 plasmid.

Restriction Endonucleases	Number of Fragments	Fragment Size (kbp)	Total Size (kbp)
Pst I / *Bam*H I	10	21.0, 3.4, 2.95, 1.7, 1.6, 1.34, 1.2, 1.02, 0.96, 0.87	36.04
Pst I / *Xba* I	10	21.0, 3.2, 2.95, 1.72, 1.62, 1.37, 1.05, 0.98, 0.87, 0.72	35.48
Pst I / *Hind* III	13	10.1, 4.3, 2.75, 2.35, 2.15, 1.85, 1.7, 1.6, 1.49, 1.34, 1.02, 0.96, 0.72	32.33
Pst I / *Sal* I	12	7.8, 5.2, 3.8, 3.2, 1.72, 1.62, 1.57, 1.37, 1.02, 0.96, 0.72, 0.59	29.57
Pst I / *Eco*R I	11	15.6, 5.0, 3.7, 2.95, 2.8, 1.72, 1.62, 1.4, 1.34, 1.02, 0.98	38.13

Hind III / *Sal* I	12	10.1, 5.9, 5.0, 3.8, 3.3, 2.68, 2.1, 1.55, 1.02, 0.98, 0.77, 0.72	37.92
Hind III / *Eco*R I	10	10.38, 9.1, 4.2, 3.25, 2.3, 2.18, 1.4, 0.94, 0.77, 0.63	35.15

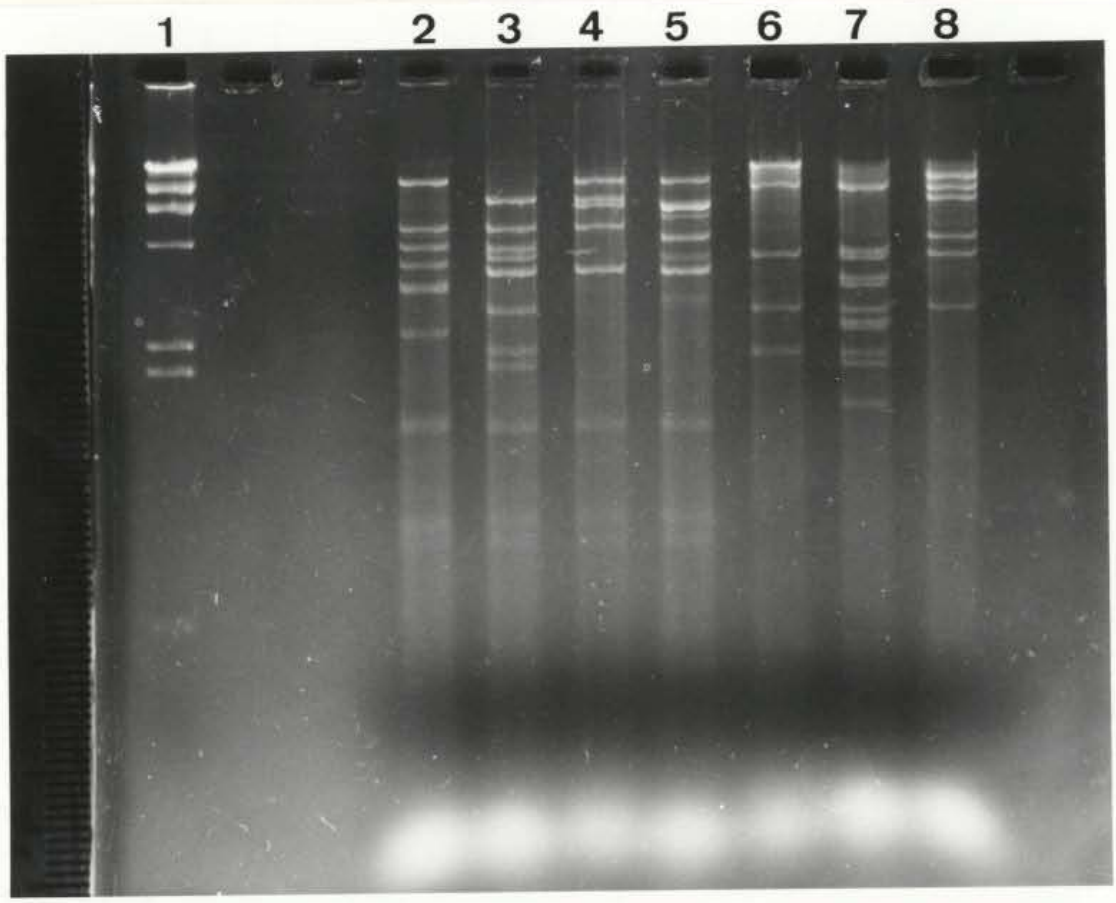

Figure 3.5. Restriction endonuclease double digest fragments of pT24. Lane 1 = *Hind* III digested λ DNA, lane 2 = *Sal* I / *Eco*R I, lane 3 = *Sal* I / *Bam*H I, lane 4 = *Sal* I / *Xba* I, lane 5 = *Sal* I / *Sac* I, lane 6 = *Bam*H I / *Xba* I, lane 7 = *Bam*H I / *Hind* IIII lane 8 = *Bam*H I / *Sac* I. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Table 3.9. Molecular weight of fragments derived from double restriction endonuclease digests of the pT24 plasmid.

Restriction Endonucleases	Number of Fragments	Fragment Size (kbp)	Total Size (kbp)
Sal I / *Eco*R I	10	9.4, 5.3, 4.4, 3.85, 3.28, 2.35, 1.55, 1.3, 1.0, 0.9	33.33
Sal I / *Bam*H I	11	7.8, 5.3, 4.3, 4.0, 3.7, 2.73, 2.1, 2.0, 1.55, 1.0, 0.9	43.48
Sal I / *Xba* I	8	9.4, 7.8, 7.0, 5.3, 3.6, 1.55, 1.0, 0.9	36.55
Sal I / *Sac* I	10	9.4, 7.2, 6.0, 5.0, 4.0, 3.7, 3.0, 1.55, 1.0, 0.9	41.75
*Bam*H I / *Xba* I	6	8.8, 6.6, 5.3, 4.1, 2.85, 2.1	29.75
*Bam*H I / *Hind* III	11	8.8, 4.2, 3.93, 3.46, 3.28, 2.73, 2.52, 2.45, 2.1, 2.0, 1.67	37.14
*Bam*H I / *Sac* I	6	12.0, 8.8, 7.2, 4.6, 4.0, 2.73	39.33

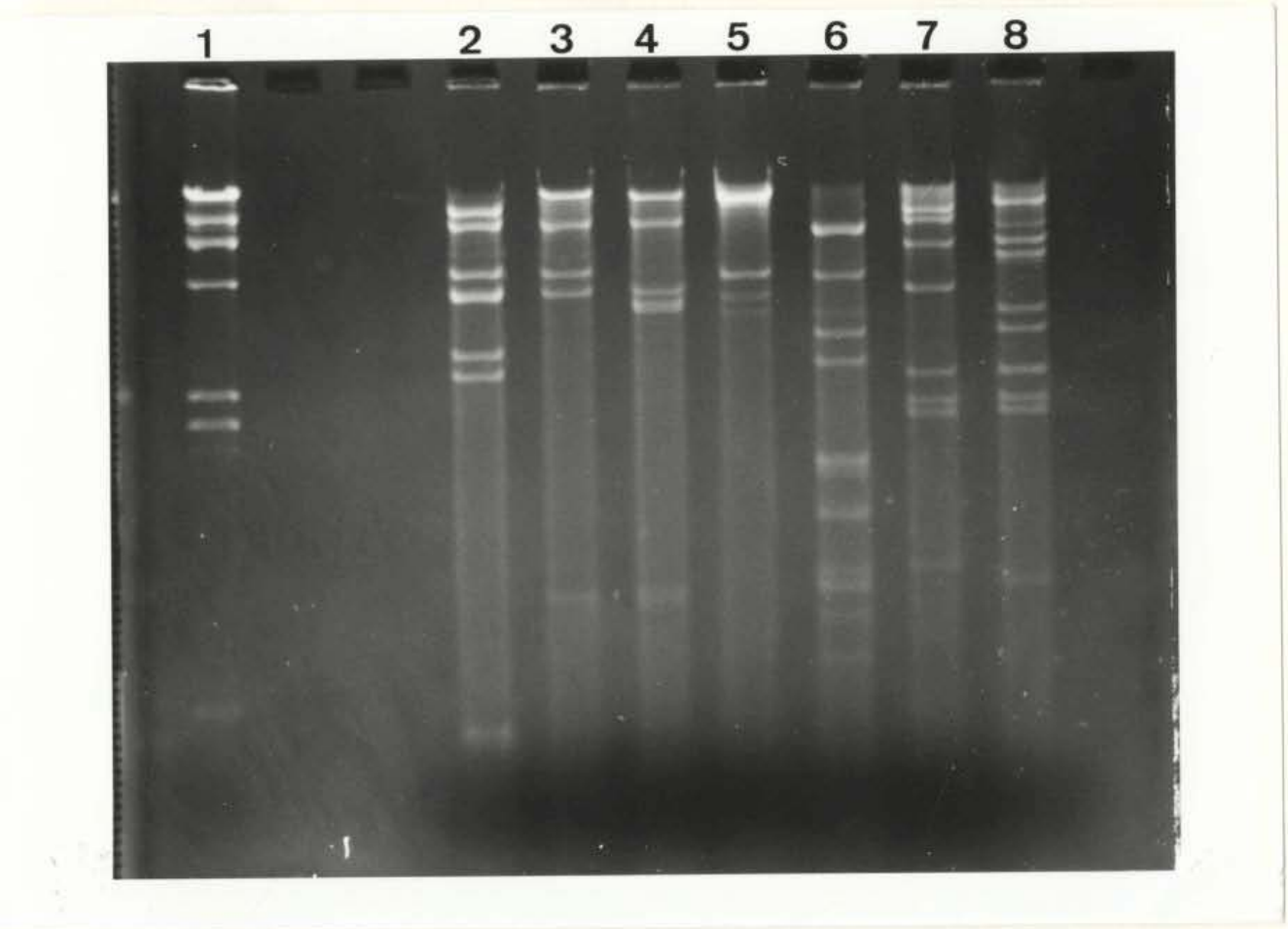

Figure 3.6. Restriction endonuclease double digest fragments of pT24. Lane 1 = *Hind* III digested λ DNA, lane 2 = *Eco*R I / *Bam*H I, lane 3 = *Eco*R I / *Xba* I, lane 4 = *Eco*R I / *Sac* I, lane 5 = *Xba* I / *Sac* I, lane 6 = *Pst* I / *Sac* I, lane 7 = *Hind* III / *Xba* I, lane 8 = *Hind* III / *Sac* I. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Table 3.10. Molecular weight of fragments derived from double restriction endonuclease digests of the pT24 plasmid.

Restriction Endonucleases	Number of Fragments	Fragment Size (kbp)	Total Size (kbp)
*Eco*R I / *Bam*H I	7	10.35, 8.4, 5.19, 4.2, 3.0, 2.6, 0.5	34.24
*Eco*R I / *Xba* I	5	23.0, 8.4, 4.9, 4.05, 0.94	41.29
*Eco*R I / *Sac* I	5	23.0, 9.0, 4.25, 3.95, 0.94	41.14
Xba I / *Sac* I	4	23.0, 5.0, 4.05, 3.8	35.85
Pst I / *Sac* I	11	7.6, 4.9, 3.35, 2.8, 1.75, 1.63, 1.35, 1.03, 0.96, 0.86, 0.7	26.93
Hind III / *Xba* I	8	12.0, 8.8, 6.4, 4.25, 2.6, 2.25, 2.2, 1.08	39.58
Hind III / *Sac* I	9	9.0, 6.0, 5.6,, 3.95, 3.5, 2.6, 2.25, 2.15, 1.0	36.05

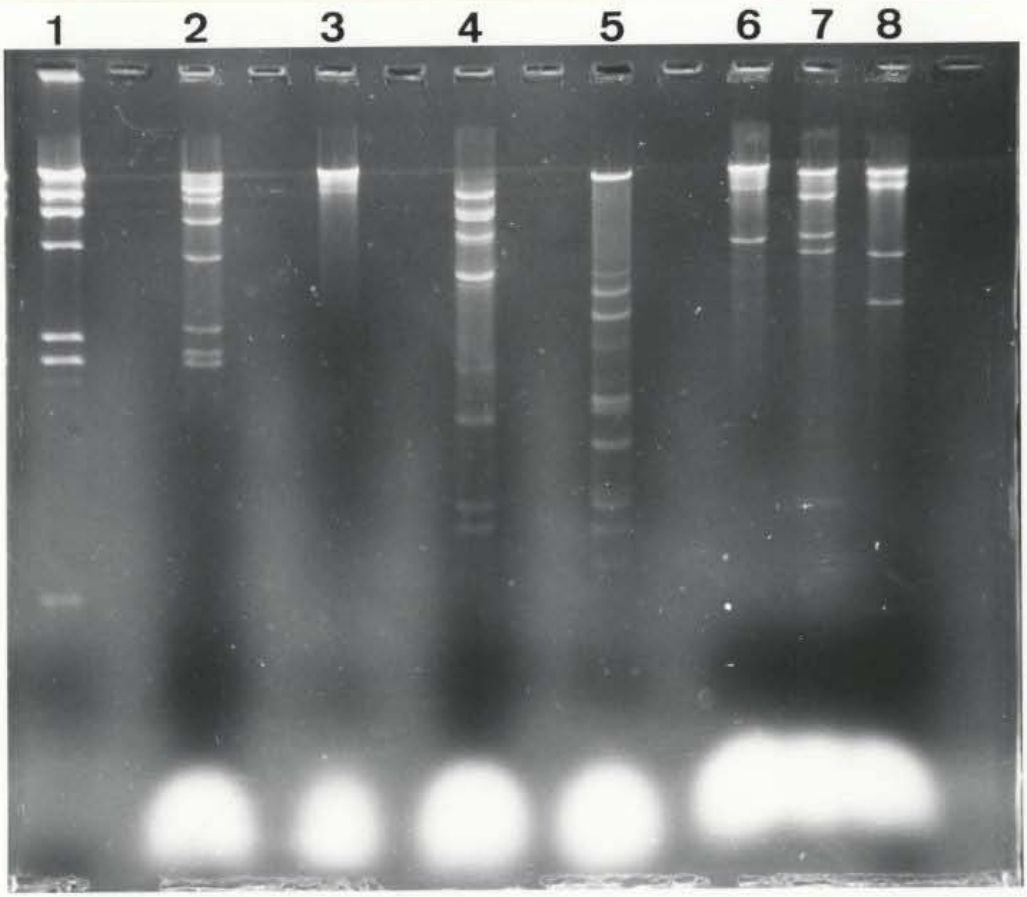

Figure 3.7. Restriction endonuclease single digest fragments of pT25. Lane 1 = *Hind* III digested λ DNA, lane 2 = *Hind* III, lane 3 = *Xba* I, lane 4 = *Sal* I, lane 5 = *Pst* I, lane 6 = *Sac* I, lane 7 = *Eco*R I, and lane 8 = *Bam*H I. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Table 3.11. Molecular weight of fragments derived from single restriction endonuclease digests of the pT25 plasmid.

Restriction Endonucleases	Number of Fragments	Fragment Size (kbp)	Total Size (kbp)
Hind III	7	12.0, 8.3, 6.2, 4.25, 2.5, 2.15, 1.94	37.34
Xba I	2	25.0, 12.0	37.0
Sal I	8	9.4, 7.4, 5.4, 3.75, 3.42, 1.45, 0.96, 0.84	32.62
Pst I	10	23.0, 3.42, 2.75, 1.7, 1.6,1.4, 1.34, 1.05, 1.0, 0.86	38.12
Sac I	2	28.0, 4.7	32.7
*Eco*R I	5	19.0, 8.3, 4.9, 4.25, 1.0	37.45
*Bam*H I	4	19.0, 10.6, 4.1, 2.8	36.5
Hind III digested λ DNA	7	23.0, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56	

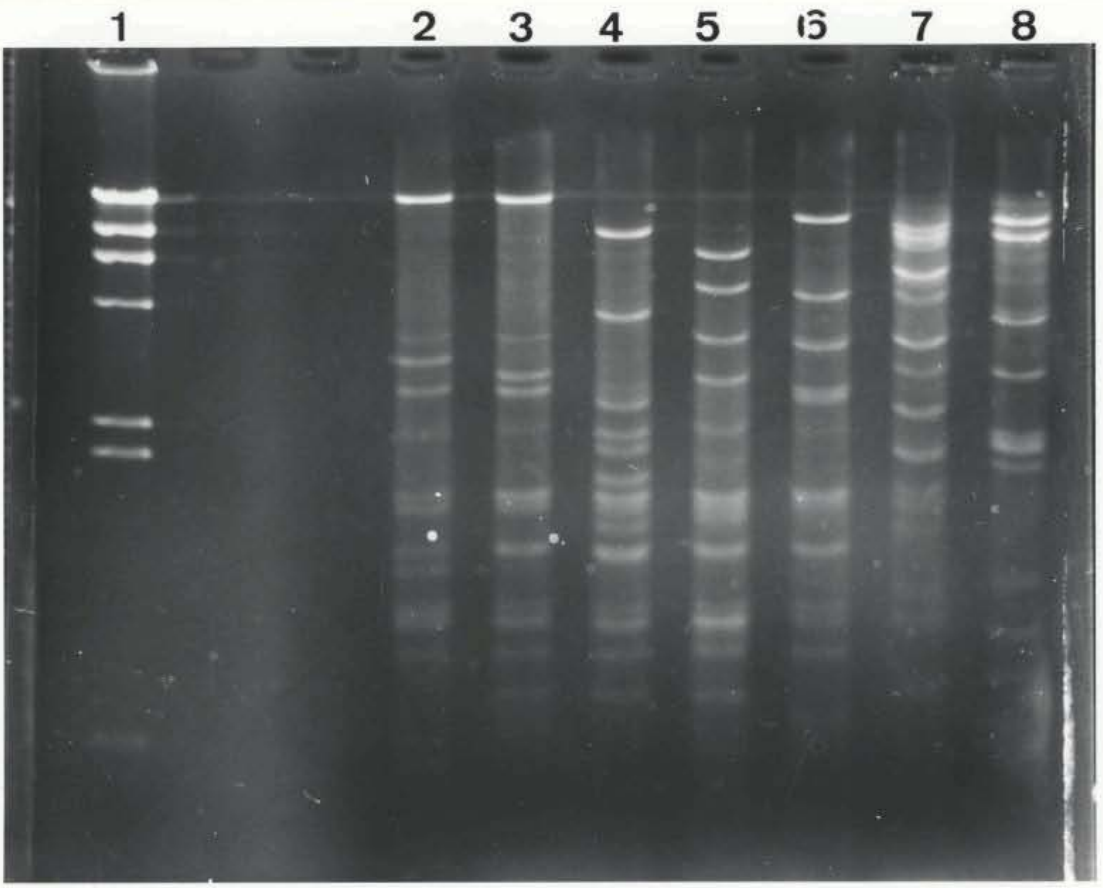

Figure 3.8. Restriction endonuclease double digest fragments of pT25. Lane 1 = *Hind* III digested λ DNA, lane 2 = *Pst* I / *Bam*H I, lane 3 = *Pst* I / *Xba* I, lane 4 = *Pst* I / *Hind* III, lane 5 = *Pst* I / *Sal* I, lane 6 = *Pst* I / *Eco*R I, lane 7 = *Hind* III / *Sal* I lane 8 = *Hind* III / *Eco*R I. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Table 3.12. Molecular weight of fragments derived from double restriction endonuclease digests of the pT25 plasmid.

Restriction Endonucleases	Number of Fragments	Fragment Size (kbp)	Total Size (kbp)
Pst I / *Bam*H I	10	23.0, 3.5, 3.2, 1.75, 1.69, 1.43, 1.3, 1.15, 1.08, 0.96	39.06
Pst I / *Xba* I	10	23.0, 3.1, 3.0, 1.8, 1.69, 1.44, 1.11, 1.08, 0.96, 0.82	38.0
Pst I / *Hind* III	14	10.18, 4.35, 2.75, 2.45, 2.15, 1.85, 1.77, 1.69, 1.53, 1.4, 1.15, 1.08, 0.98, 0.82	34.15
Pst I / *Sal* I	12	6.6, 5.35, 3.9, 3.2, 1.77, 1.69, 1.60, 1.45, 1.18, 1.1, 0.98, 0.82	29.61

Pst I / *Eco*R I	11	16.5, 5.0, 3.81, 3.3, 3.15, 1.85, 1.77, 1.47, 1.18, 1.11, 0.98	40.12
Hind III / *Sal* I	11	9.4, 7.0, 6.0, 4.0, 3.85, 2.4, 2.0, 1.0, 0.9, 0.72, 0.7	37.97
Hind III / *Eco*R I	7	12.5, 10.18, 4.35, 3.4, 2.5, 2.09, 1.3	36.32

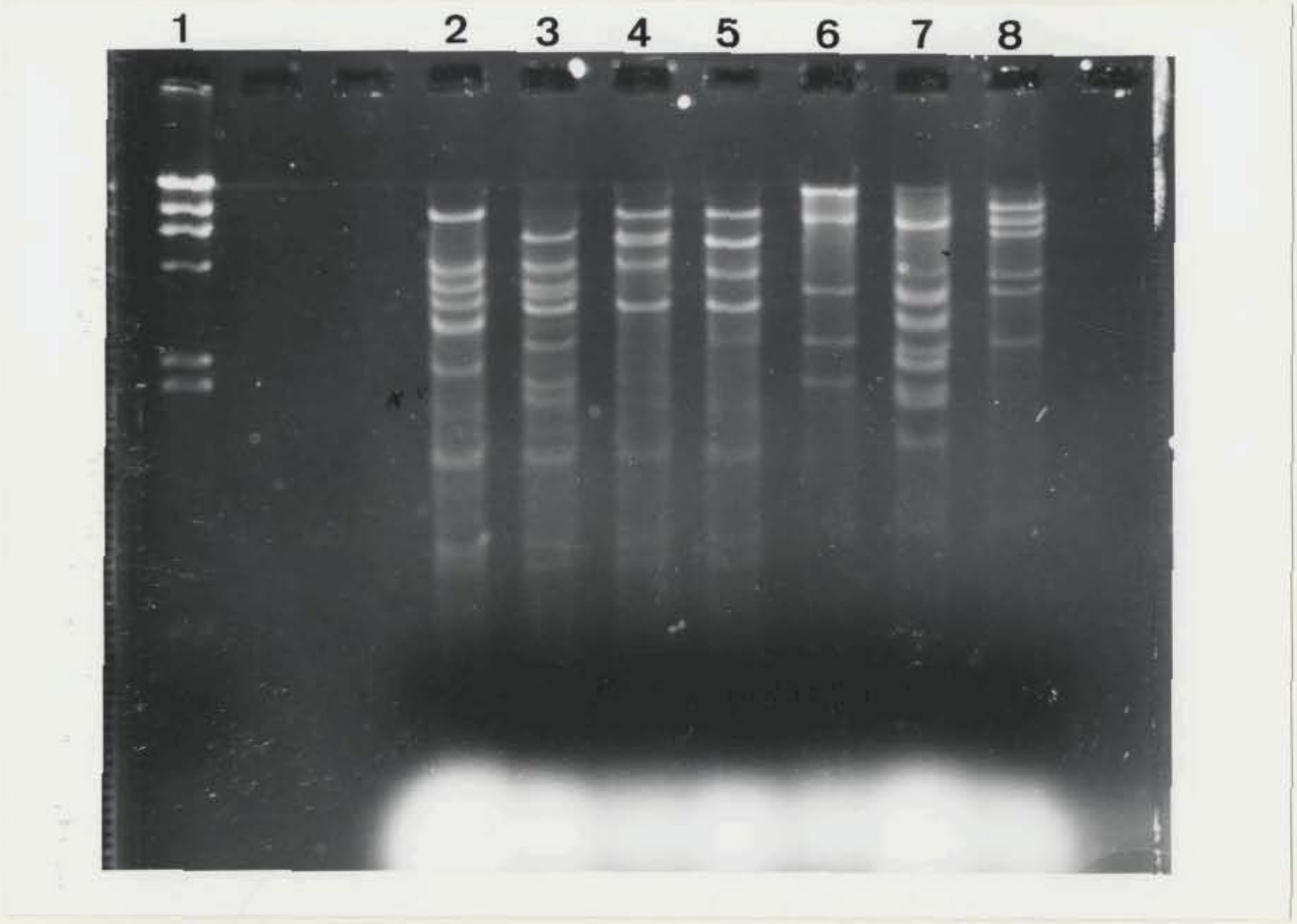

Figure 3.9. Restriction endonuclease double digest fragments of pT25. Lane 1 = *Hind* III digested λ DNA, lane 2 = *Sal* I / *Eco*R I, lane 3 = *Sal* I / *Bam*H I, lane 4 = *Sal* I / *Xba* I, lane 5 = *Sal* I / *Sac* I, lane 6 = *Bam*H I / *Xba* I, lane 7 = *Bam*H I / *Hind* III, lane 8 = *Bam*H I / *Sac* I. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Table 3.13. Molecular weight of fragments derived from double restriction endonuclease digests of the pT25 plasmid.

Restriction Endonucleases	Number of Fragments	Fragment Size (kbp)	Total Size (kbp)
Sal I / *Eco*R I	10	8.2, 4.2, 3.7, 3.32, 2.81, 2.1, 1.4, 1.34, 0.84, 0.77	28.68
Sal I / *Bam*H I	11	6.6, 4.6, 3.95, 3.7, 3.32, 2.6, 2.0, 1.45, 1.4, 0.89, 0.8	31.31
Sal I / *Xba* I	9	8.2, 6.6, 6.2, 5.0, 3.45,1.45, 1.4, 0.92, 0.84	34.06
Sal I / *Sac* I	9	8.2, 6.2, 4.3, 3.45, 2.7, 1.45, 1.4, 0.92, 0.8	29.42
*Bam*H I / *Xba* I	5	12.0, 8.2, 3.7, 2.7, 2.0	28.6
*Bam*H I / *Hind* III	9	8.2, 4.4, 4.0, 3.2, 2.6, 2.3, 2.0, 1.87, 1.53	30.1

*Bam*H I / *Sac* I	6	10.15, 8.6, 6.6, 4.3, 3.7, 2.7	36.05

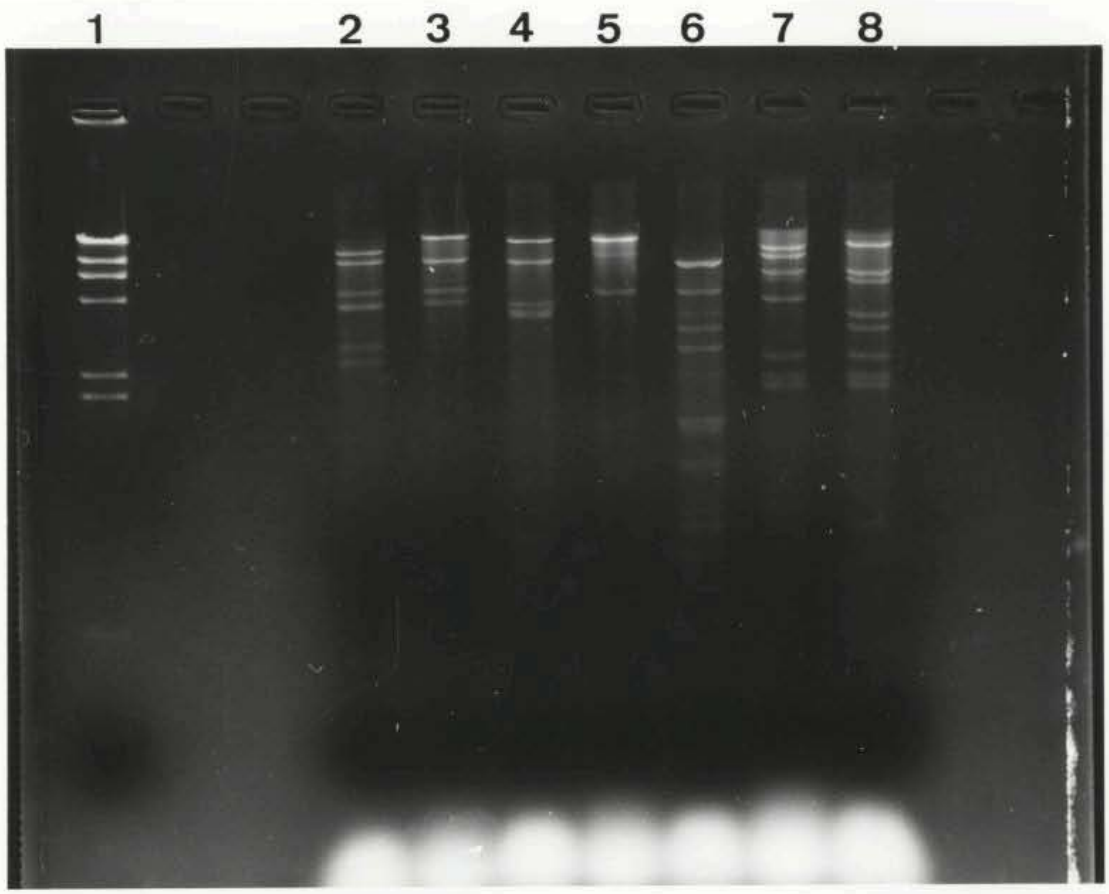

Figure 3.10. Restriction endonuclease double digest fragments of pT25. Lane 1 = *Hind* III digested λ DNA, lane 2 = *Eco*R I / *Bam*H I, lane 3 = *Eco*R I / *Xba* I, lane 4 = *Eco*R I / *Sac* I, lane 5 = *Xba* I / *Sac* I, lane 6 = *Pst* I / *Sac* I, lane 7 = *Hind* III / *Xba* I, lane 8 = *Hind* III / *Sac* I. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Table 3.14. Molecular weight of fragments derived from double restriction endonuclease digests of the pT25 plasmid.

Restriction Endonucleases	Number of Fragments	Fragment Size (kbp)	Total Size (kbp)
*Eco*R I / *Bam*H I	6	10.5, 9.4, 4.25, 3.85, 2.95, 2.55	33.5
*Eco*R I / *Xba* I	4	23.0, 9.4, 4.25, 4.0	40.65
*Eco*R I / *Sac* I	4	23.0, 9.4, 3.85, 3.65	39.9
Xba I / *Sac* I	2	23.0, 4.8	27.8
Pst I / *Sac* I	11	8.6, 4.25, 3.35, 2.85, 1.77, 1.7, 1.46, 1.41, 1.02, 0.9, 0.74	28.05
Hind III / *Xba* I	8	10.5, 7.7, 6.8, 4.1, 2.7, 2.4, 2.2, 1.13	37.53
Hind III / *Sac* I	9	9.0, 5.9, 5.6, 3.6, 3.3, 2.7, 2.22, 2.12, 1.0	35.44

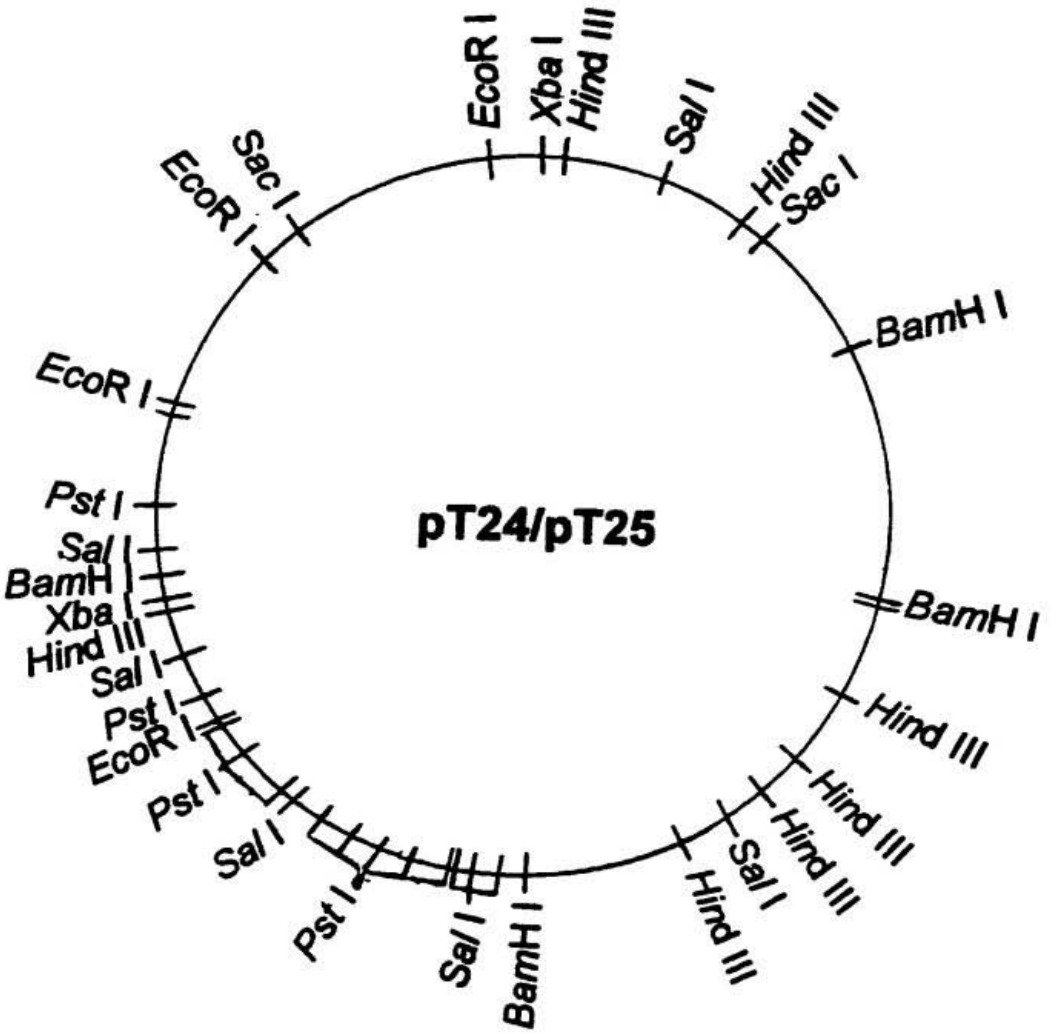

Figure 3.11. Restriction endonuclease digest map for the plasmids pT24 and pT25

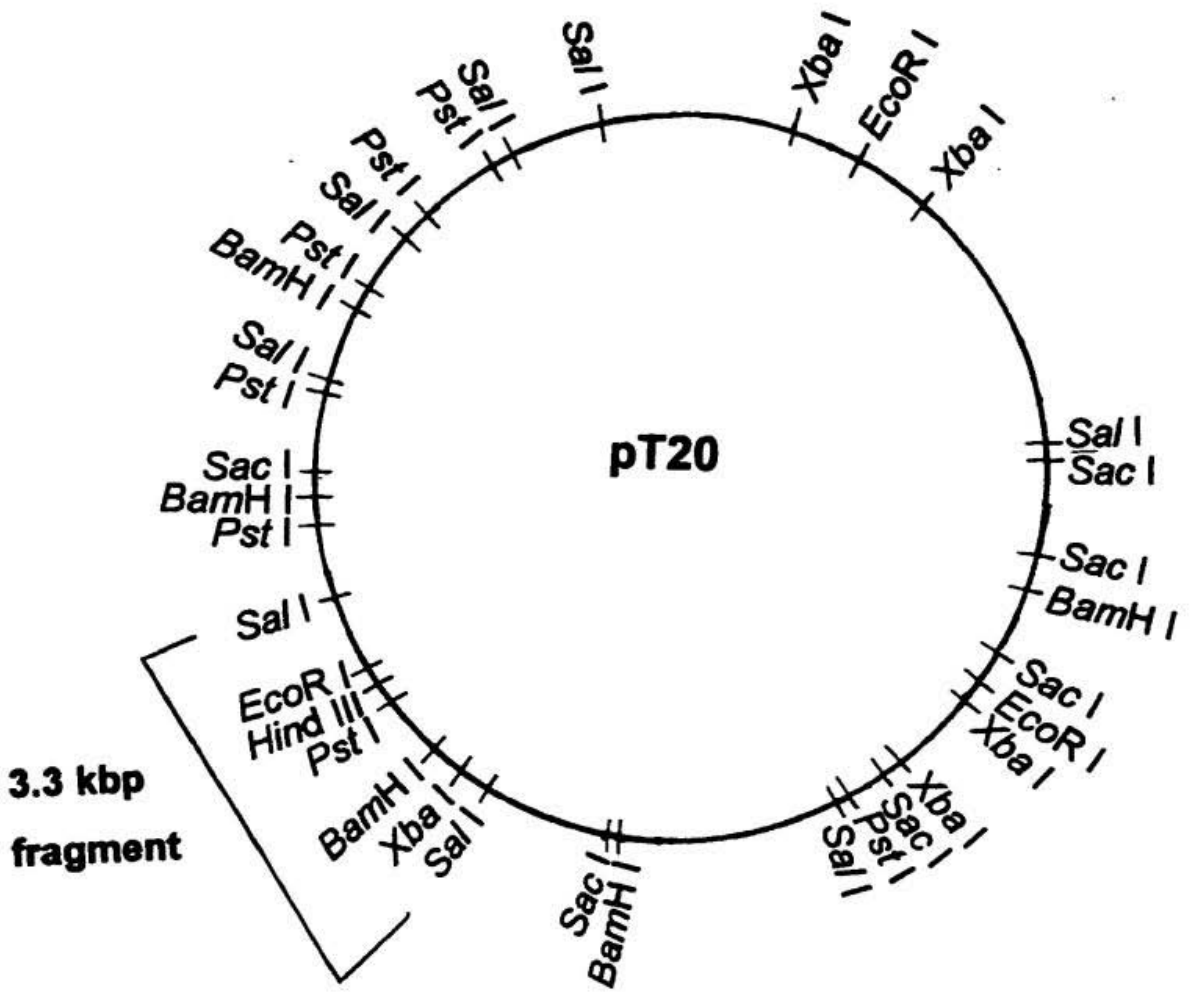

Figure 3.12. Restriction endonuclease digestion map of pT20; modified version of that presented by Hatfield (1990).

3.6. Probing of pT24 and pT25

Single restriction endonuclease digests of pT24 and pT25 (fig.3.13 and 3.15) were transferred onto positively charged nylon membranes and were probed with the DIG-labeled pUT 8 construct. Figure 3.14 illustrates that the pT24 had regions of similarity to the DIG-labeled pUT 8 construct. The detected regions of similarity were: the 20 kbp fragment of the *Bam*H I digest, the 20 kbp fragment of the *Eco*R I digest, the 28 kbp fragment of the *Sac* I digest, the 3.4 kbp and 1.72 kbp fragments of the *Pst* I digest, the 3.75 kbp fragment of the *Sal* I digest, the 23 kbp fragment of the *Xba* I digest, and the 8.2 kbp fragment of the *Hin*d III digest. Figure 3.16 illustrates that the pT25 had regions of similarity to the DIG labeled pUT 8 construct. The detected regions of similarity were: the 19 kbp fragment of the *Bam*H I digest, the 19 kbp fragment of *Eco*R I digest, the 23 kbp fragment of the *Sac* I digest, the 3.42 kbp and the 1.7 kbp fragments of the *Pst* I digest, the 3.75 kbp fragment of the *Sal* I digest, the 25 kbp fragment of the *Xba* I digest, and the 8.3 kbp fragment of the *Hin*d III digest. The regions of similarity between the pUT 8 construct and the two plasmids produced a similar pattern, as indicated by figures 3.14 and 3.16.

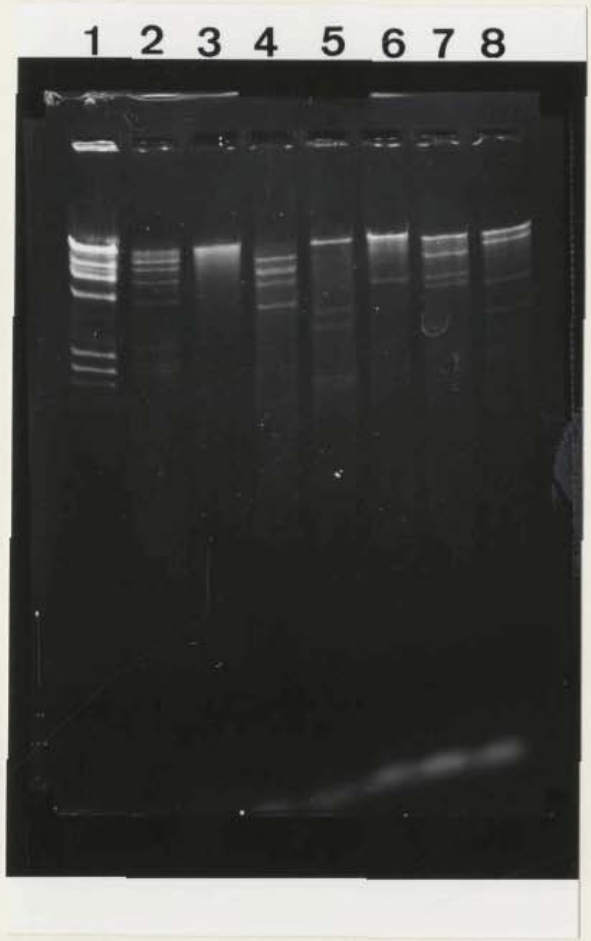

Figure 3.13. Single restriction endonuclease digest fragments of pT24. Lane 2 = *Hind* III, lane 3 = *Xba* I, lane 4 = *Sal* I, lane 5 = *Pst* I, lane 6 = *Sac* I, lane 7 = *Eco*R I, lane 8 = *Bam*H I, and lane 1 = *Hind* III digested λ DNA. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Figure 3.14. Southern blot of fragments generated from the single digests of pT24 probed with the DIG labeled pUT 8 construct. Lane 1= *Bam*H I digest, lane 2= *Eco*R I digest, lane 3= *Sac* I digest, lane 4= *Pst* I digest, lane 5= *Sal* I digest, lane 6= *Xba* I digest, lane 7= *Hin*d III digest, and lane 8= *Hin*d III digested λ DNA. (Inverse of agarose gel image).

Figure 3.15. Restriction endonuclease single digest fragments of pT25. Lane 2 = *Hind* III, lane 3 = *Xba* I, lane 4 = *Sal* I, lane 5 = *Pst* I, lane 6 = *Sac* I, lane 7 = *Eco*R I, and lane 8 = *Bam*H I, and lane 1 = *Hind* III digested λ DNA. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

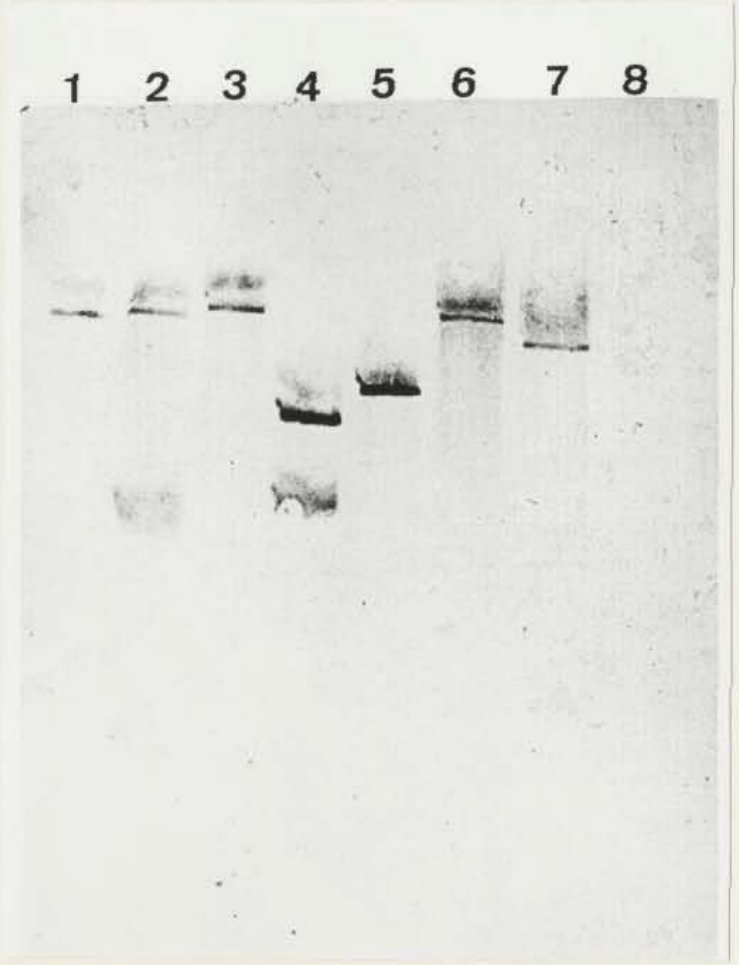

Figure 3.16. Southern blot of fragments generated from the single digests of pT25 probed with the DIG labeled pUT 8 construct. Lane 1= *Bam*H I digest, lane 2= EcoR I digest, lane 3= *Sac* I digest, lane 4= *Pst* I digest, lane 5= *Sal* I digest, lane 6= *Xba* I digest, lane 7= *Hin*d III digest, and lane 8= *Hin*d III digested λ DNA. (Inverse of agarose gel image).

3.7. Probing the genomic DNA from *P. fluorescens*, strains T24 and T25

The *Bam*H I digested genomic DNA, from both strains T24 and T25, and plasmid DNA, from strains T24 and T25, were transferred onto a positively charged nylon membrane via the method of Southern and probed with the DIG labeled pUT 8 construct. The pUT 8 construct had regions of similarity with the plasmids, but not with the genomic DNA digests, as indicated in figure 3.18.

3.8. Subcloning of DNA fragments

Various ligation reactions were conducted between digested pUC 18 and digested pT24 and pT25, respectively, and the ligation products were used to transform competent *E. coli* , strain DH1. In addition, the 3.4 kbp *Pst* I fragment from pT24 and the 3.75 kbp *Sal* I fragment from pT25, were also ligated with the corresponding digested pUC 18 and used to transform DH1. As indicated in table 3.15, no protease positive transformants were detected. The *Bam*H I digests of the genomic DNA from *P. fluorescens* strains T24 and T25 were also ligated with *Bam*H I digested pUC 18 and introduced into competent DH1 . No protease positive transformants were detected.

Figure 3.17. Plasmid and digested genomic DNA from *P. fluorescens*, strains T24 and T25. Lane 4= pT24, lane 5= *Bam*H I digested genomic DNA from strain T24, lane 6= pT25, lane 7= *Bam*H I digested genomic DNA from strain T25, and lane 2= *Hind* III digested λ DNA. Electrophoresis was conducted using a 0.8% agarose, and the gel was run for approximately 2 hr at 80 volts.

Figure 3.18. Southern blot of *Bam*H I digested genomic DNA from *P. flourescens*, strains T24 and T25, and pT24 and T25, probed with the DIG labeled pUT 8 construct. Lane 2 = *Bam*H I digested genomic DNA from strain T25, lane 3 = pT25 , lane 4 = *Bam*H I digested genomic DNA from strain T24, lane 5 = pT24 and lane 7 = Hind III digested λ DNA. (Inverse of the agarose gel image).

Table 3.15. Transformation experiments using various ligation mixtures containing pUC 18 and either plasmid DNA or genomic DNA from *P. fluorescens*, strains T24 and T25, and competent *E. coli* strain DH1.

Ligation Reaction Mixture	Number of Colonies Screened	Number of Protease Positive Colonies Detected
3.4 kbp *Pst* I fragment (pT24) / *Pst* I digested pUC 18	8307	0
3.75 kbp *Sal* I fragment (pT25) / *Sal* I digested pUC 18	5005	0
Pst I digested pT24 / *Pst* I digested pUC 18	3703	0
Sal I digested pT25 / *Sal* I digested pUC 18	5225	0
*Bam*H I digested genomic T24 / *Bam*H I digested pUC 18	5202	0
*Bam*H I digested genomic T25 / *Bam*H I digested pUC 18	6404	0

Chapter 4

Discussion

Protease location

Conjugation and transformation experiments were employed to determine whether or not the protease genes of *P. fluorescens* strains T24 and T25 were located on the plasmid DNAs present. Protease positive transconjugants and transformants were not detected, which neither supports nor disproves the hypothesis that the protease genes are located on the plasmid DNAs.

The absence of transconjugant *E. coli* colonies containing the plasmid DNA encoding for the extracellular protease may have been due to repression of transfer of the plasmid from *P. fluorescens*. According to Provence and Curtiss (1994), most strains of bacteria found in nature have repressed conjugative plasmids, such that only 1 in 10^3 to 10^4 cells containing the conjugative plasmid are able to transfer it. In the experiments reported here, it is possible that the frequency of transfer may have been below the limit of detection (Willetts 1993). Derepression can occur on transfer of the plasmid to a recipient cell; to detect the transfer of a conjugative plasmid, mating may need to be conducted over a period of several hours or longer (Provence and Curtiss 1994). This factor was

taken into consideration in both the filter and plate mating protocols, which were conducted for 24-48 hrs, but protease positive transconjugants were still not detected under these conditions

Another factor that plays an important role in the transfer efficiency of conjugative plasmids is the mating environment. Some plasmids promote conjugational DNA transfer more efficiently when the donor and recipient cells are mated on a solid surface, such as on agar plates or on a membrane filter (Provence and Curtiss 1994), whereas other plasmids are more efficiently transferred in liquid media (Provence and Curtiss 1994). The preference for the mating medium is determined by the type of pili synthesized; flexible pili transfer equally well in both broth and solid surface matings, but rigid pili only transfer efficiently when the matings are conducted on a solid surface (Willetts 1988). Willetts (1988) indicated that, of the mating techniques, the membrane filter mating technique was perhaps the most efficient followed by plate mating, then broth mating. *Pseudomonas* plasmids have a surface mating preference or are capable of transfer equally well in liquid and on solid surfaces (Bradley 1983). Three different methods of conjugation were conducted and of the thousands of colonies screened, no protease positive transconjugants were detected.

Temperature is another important parameter to be considered when conducting conjugation experiments. Provence and Curtiss (1994) recommend that conjugation experiments be conducted at both 25°C and 37°C, when

examining whether a trait might be transferred on a conjugative plasmid. Both temperatures were employed in the experiments.

Low levels or inefficient conjugation could also be due to the presence of fertility inhibition (*fin*) genes located on the plasmid. According to Willetts (1988), *fin* genes may control the expression of some, but not all, plasmid conjugation systems. For example, two *fin* genes, *fin O* and *fin P*, are found in most of the IncF (incompatibility group F) plasmids. The products of these two genes together prevent the transcription of a positive control gene, *tra J*, necessary for the transcription of all other transfer genes. Consequently, cells carrying the wild-type IncF plasmids can only transfer them at about 0.1% of the maximal frequency.

Although conjugation has been reported to occur between *P. fluorescens* strains (donor) and *E.coli* strains (recipient), the relatedness of the two groups may play a role in the determination of conjugation. The interactions between the donor cell pili and the recipient cell receptors is an important aspect of many conjugal transfers, and may limit the host range of the conjugal gene transfer (Harwood 1993).

It is also possible that the plasmid DNA was transferred from the *P. fluorescens* strains to the *E. coli* strains, and the transconjugants were not detected. The selection criterion used to detect the transconjugant colonies was the expression of the protease gene in the transconjugant; as indicated by the

degradation of milk powder in the agar plates. The transcription-translation machinery of *E. coli* does not always recognize well the transcription-translation signals from many other species. Consequently, the *Pseudomonas* genes may have been expressed poorly, if at all, under the regulatory signals present in *E. coli* (Morales et al. 1990). Therefore, if transconjugant *Pseudomonas* protease genes were not expressed in *E. coli*, they would not have been detected by the assay used.

The experimental protocol assumed that the plasmids present in *P. fluorescens* strains T24 and T25 were conjugative plasmids. John et al. (1981) indicated that many naturally occurring plasmids are unable to transfer themselves to other cells. Most such nonconjugative plasmids are small and lack the *tra* genes required for synthesis of pili (Willetts 1988). However, many of these plasmids contain the *bom* locus, with an *ori T* sequence, from which the conjugal transfer can be initiated (Provence and Curtiss 1994; Willetts 1988). Some of these plasmids are capable of conjugal transfer, by using the *tra* gene products encoded by another conjugative plasmid, which is referred to as a mobilization plasmid (mob^+) (Provence and Curtiss 1994; Harwood 1993). Not all conjugative plasmids will mobilize a given nonconjugative plasmid, and several different conjugative plasmids would have to be tested for its mobilization ability (Willetts 1988).

The effectiveness of artificial transformation, with regards to the uptake of plasmid and chromosomal DNA is not clearcut. Some researchers suggest that transformation is more effective with plasmid DNA than with chromosomal DNA, because plasmid DNA is not as readily degraded as chromosomal DNA, and can also replicate within the host (Prescott et al. 1990; Carlson et al 1984). However, Mazodier and Davies (1991) indicate that transformation with plasmid DNA may sometimes be less effective than with chromosomal DNA. Therefore, it is not clear whether or not the use of plasmid DNA, could have affected the effectiveness of the transformation in this experiment. It has also been found that transformation with plasmids larger than 15 kbp, is often less effective than that with smaller plasmids (Saunders and Saunders 1988). Therefore, the size of the plasmids used here (~ 40 kbp) may have played an important role in the lack of success of the transformation experiments.

There are a number of other important parameters that can also affect the success of the transformation. First, there are various aspects of the bacterial culture which can be of great importance. The genotype of the bacterial culture is quite important in determining whether the introduced DNA persists and is stable after entry into the cell (Provence and Curtiss 1994). The *E. coli* strains chosen are those that are restriction deficient, and have been successfully employed in various transformation experiments (Singer and Berg 1991; Miller et al. 1988). The growth conditions used to make the recipient cell receptive, are

important and can affect the efficiency of DNA entry into the cell, especially with the $CaCl_2$ artificially induced competent cells (Provence and Curtiss 1994). However, the *E. coli* cells prepared for both transformation techniques were transformed efficiently by pUC 18 (3.4×10^5 transformants per µg of DNA with $CaCl_2$ and 4.42×10^6 transformants per µg of DNA with electroporation). With the electroporation technique there are additional technical factors, such as the choice of the appropriate voltage, pulse and duration, which are also critical to the effectiveness of the transformation (Provence and Curtiss 1994; Miller et al. 1988). The parameters used were able to successfully transform the *E. coli* cells with the pUC 18 plasmid, but they may not have been ideal for the pT24 and pT25 plasmids.

Both the presence and percentage of cured *P. fluorescens* strain T25 colonies, following heat treatment, indicate that the protease gene(s) is possibly located on the extracellular plasmid DNA present in this bacterial strain. The results can be attributed to the heat treatment and not to spontaneous mutations, which usually occur at very low frequencies (Eisenstadt et al. 1994). Stanisich's (1988) suggestion that the loss of a property, such as protease activity, may be due to an induced gene mutation, must also be excluded. The high percentage of cured colonies, the reculturing of the protease negative colonies, and particularly the absence of plasmid DNA in these colonies,

indicates that the protease gene(s) was lost due to curing of the plasmid DNA and not due to gene mutation. In addition, the series of biochemical tests, gram stain, fluorescent pigment production and growth temperature tests, all confirmed that the protease negative colonies are *P. fluorescens*.

The apparent reversion of some of the colonies from protease negative to protease positive may be explained in a number of ways. First, the protease negative colony may have been contaminated with protease positive bacteria, which subsequently, gave the protease activity upon replating. Second, the regulatory mechanism for the protease gene(s) of *P. fluorescens* strain T25 is unknown, and the elevated temperature may have had an adverse effect on the regulation and expression of the protease gene(s) temporarily, in some of the colonies. However, when the colonies were cultured at the optimum growth temperature, the protease gene(s) was expressed. The third possible explanation deals with the nature of the plasmid DNA itself. Very little is known about the plasmid from *P. fluorescens* strain T25, and the possibility exists that it is an episome, capable of integration into the chromosomal DNA. In the protease negative colonies, the episome may have been integrated into the chromosomal DNA in such a way that the protease gene(s) was not expressed. However, upon replating, the episome may have excised itself from the chromosomal DNA, thus allowing the expression of the protease gene(s). Finally, the revertant colonies may also indicate that the original loss of protease

activity, in these colonies, was due to a plasmid-associated gene mutation rather than the loss of the plasmid (Stanisich 1988). The revertant colonies were not viable and further testing could not be conducted.

The attempt, via electroporation, to reintroduce the plasmid DNA into the cured *P. fluorescens* strain T25 colonies did not produce any protease positive transformants. However, there were only approximately 3500 colonies screened and this may have not been enough to detect any possible transformants. In addition, electroporation is not always successfully able to transform bacteria with plasmid DNA (Miller et al. 1996).

Relatively little research has been conducted to determine the genetic location of protease gene(s) in pseudomonad species in general. Current research has determined that an alkaline serine protease gene from an alkaline resistant *Pseudomonas sp.* is located on the chromosomal DNA (Jang et al. 1996). However, the majority of research has been conducted on medically significant strains of *P. aeruginosa*, in which the protease gene is also located on the chromosomal DNA (Bally et al. 1989). Hence, the possibility that the protease gene(s) of *P. fluorescens* strain T25 is located on plasmid DNA, could be quite novel.

The absence of cured protease negative colonies of *P. fluorescens* strain T24 following heat treatment is not evidence against plasmid involvement, but may simply indicate that this plasmid is refractory to the heat treatment.

The fact that protease negative, plasmid free colonies of *P. fluorescens* strain T25 were produced, does not mean that the same treatment would also successfully cure the T24 strain of its plasmid. Research has indicated that the efficiency of curing can vary widely (<0.1% - 100%) depending on both the plasmid and the particular bacterial host (Stanisich 1988). In addition, previous curing experiments conducted on *P. fluorescens* strain T20, using elevated temperatures, were also unsuccessful at producing protease negative, plasmid free colonies (Baksh 1992; Wells 1994).

Plasmid mapping

A restriction endonuclease digest map was constructed for the plasmids pT24 and pT25, from *P. fluorescens*, strains T24 and T25 respectively, using a combination of single and double restriction endonuclease digestions. The enzymes used for the pT20 plasmid map were also used to produce the map of pT24 and pT25 so that comparisons of the plasmids could be made. The map produced for the two plasmids differs from that of the pT20 plasmid from *P. fluorescens* strain T20, indicating that they are two different plasmids.

The number and size of fragments produced by the digestions of both the pT24 and pT25 plasmids are not identical. For example the single restriction endonuclease *Sac* I digest for pT24 has 2 fragments that are 28.0 kbp and 5.6 kbp in size, while the same digest for pT25 was reported to produce a 28.0 kbp

fragment and a 4.7 kbp fragment (Tables 3.7 and 3.11), and for the *Eco*R I digest for pT24 there are 5 fragments visible in figure 3.3, but there are only 4 visible for pT25 in figure 3.7. These differences can be accounted for in a number of ways. First, some of the enzymes such as *Xba* I and *Sac* I produced incomplete digestions, resulting in a different number of fragments for the plasmids pT24 and pT25. However, observation of some of the double digests, such as the *Xba* I and *Sac* I digest in figure 3.6, quickly revealed and alleviated some of the inconsistencies. The loading buffer front hindered the visualization of some of the bands (Fig. 3.9 and 3.10). The number of fragments for some of the double digests was not the sum of the single digests of both enzymes, as it should have been (Maniatis et al. 1982; Ausubel et al. 1995). The lower than expected number of fragments may have been due to fragments of similar length that were co-migrating and undetected. Errors in the sizing of the large fragments using the λ DNA marker was also possible. Alternatively, the apparent inconsistencies in the sum of the fragment lengths may have been due to very small fragments that may have run off of the gel or fragments that were too small to have been visualized on the 0.8% agarose gel, which can resolve DNA fragments as small as 0.5 kbp (Maniatis et al. 1982).

To produce a restriction endonuclease digestion map of an unknown plasmid, the most common approach is to use single and combination digests, using enzymes that cleave the DNA relatively infrequently. However, according

to Ausubel et al. (1995), mapping of plasmids larger than 20 kbp in length becomes progressively more difficult with this technique.

Other approaches to the construction of a restriction endonuclease digestion map are available. One alternative is the partial cleavage of radiolabeled DNA by a restriction endonuclease. The DNA of choice is linearized by digestion with an enzyme, and the ends of the DNA are then radiolabeled. One of the radiolabeled ends can then be removed by digestion with another enzyme. The DNA can then be cleaved by an enzyme of choice, and the fragments separated on an agarose gel by electrophoresis. The sizes of the fragments reflect the distance between the restriction site and the radiolabeled end of the DNA (Ausubel et al. 1995; Maniatis et al. 1982). A second alternative is the partial digestion with a nuclease, such as *Bal* 31 followed by the digestion with restriction endonucleases. In linear duplex DNA, *Bal* 31 degrades both the 5' and 3' termini, at both ends, resulting in a controlled shortening of the DNA. The reaction can be stopped at different time intervals, the DNA purified, digested with the restriction endonuclease, and run on an agarose gel. By comparison with the DNA digested only with the restriction endonuclease, the order in which the fragments disappear from the gel indicates the order in which they occur from the ends of the DNA and a map can be constructed (Ausubel et al. 1992; Legerski et al. 1978). Both of these protocols are much more time consuming than single and combination restriction

endonuclease digestions.

Southern blotting and probing of the single restriction endonuclease digests of the pT24 and pT25 plasmids with the DIG-labeled pUT8 construct, indicated that there is similarity between the plasmids and the construct supporting the hypothesis that the protease genes are located on the plasmid DNAs of *P. fluorescens* strains T24 and T25. Some of the plasmid fragments that showed similarity to the pUT 8 construct were chosen for cloning and transformation experiments. The *Sal* I 3.75 kbp fragment was chosen due to its similarity to the *Sal* I 3.3 kbp fragment of pT20 that was shown to contain a protease gene (Hatfield 1990). A second fragment, the *Pst* I 3.4 kbp fragment, was chosen on the basis of its size and similarity to the pUT 8 construct, although it was possible that it only contained a portion of the protease gene and thus would not have been expressed. Cloning of these fragments and their expression in *E. coli* was not attained. The absence of protease positive transformed *E. coli* colonies indicates that a larger number of colonies need to be screened in future experiments.

Prior to the use of the pUT8 construct, it was digested with a series of restriction endonucleases, and the restriction digests were found comparable to that attained by Hatfield (1990). However, upon later investigation, it was noted that the construct was not expressing the protease gene in *E. coli*, species DH5α or DH1, in contrast to Hatfield's report (1990). It is suggested that a

possible alternative, for future investigation of these plasmids, would be the sequencing of the 3.3 kbp insert of pUT 8 and one of the smaller fragments (*Pst* I or *Sal* I) obtained from the restriction endonuclease digests of the plasmids, that is similar to the pUT8 construct. Comparisons could then be made to any prokaryotic protease sequences located in the nucleic acid sequence databases. Consequently, it maybe determined whether or not the fragments do contain a protease gene.

The absence of sequence similarity between the pUT8 construct and the *Bam*H I digests of the genomic DNA from *P. fluorescens*, strains T24 and T25, indicates that the protease gene proposed to be located in the construct is not located in the chromosomal DNA. However, until the sequence of the 3.3 kbp insert of the pUT8 construct or the pT24 or pT25 plasmid fragments have been determined and used to verify whether or not there is a protease gene present, the lack of similarity between the chromosomal DNA and the pUT8 construct cannot be used to confirm that the protease genes are not located on the chromosomal DNA.

There are a number of considerations that could be addressed by future investigations. The genetic marker used to determine whether or not the plasmid DNA, or a fragment of the plasmid DNA hypothesized to contain the protease gene(s), was present was protease activity. These experiments

assumed that the protease gene(s) could be expressed in the host cell. It has however been reported that the transcription-translation machinery found in *E. coli* often does not recognize, or not recognize well, the transcription-translation signals found in other species (Morales et al. 1990). Therefore, if the plasmid DNA from strains T24 and T25 may have been present in the host cells, but because the protease genes were not expressed, the presence of the plasmid DNA was not detected. Similarly, the function of some operons or individual genes are related to or dependent on other biological properties of the host. When the DNA is present in *E. coli*, it may not encode the functions required for the expression of the properties associated with the DNA (Morales et al. 1990). Therefore, future investigations should consider the use of a second marker such a the differences between strains T24 and T25 and their sensitivity to chloramphenicol and streptomycin.

A second consideration is the purity of the plasmid DNA. In this experiment the plasmid DNA was isolated from *P. fluorescens*, strains T24 and T25, using the alkaline lysis protocol of Maniatis et al. (1982). The quantity of plasmid DNA present in the strains was quite small, and the plasmid was not amplified with the addition of chloramphenicol. While purifying the plasmid DNA using cesium chloride gradients, it would often become lost in the extraction solutions. The impurity of the plasmid DNA may have resulted in problems during the restriction endonuclease digests. Therefore, it is

recommended that future experiments consider the copy number of the plasmid and methods by which the plasmid could be amplified and purified, prior to restriction endonuclease digestion.

Finally, the fact that protease positive transformants, containing the pT24 and pT25 fragments ligated to the pUC 18 vector, were not detected should be addressed. The plasmid DNA fragments were similar to the pUT8 construct indicated to contain protease genes (Hatfield 1990). Therefore, the sequence of the fragments should be determined and compared to those of prokaryotic proteases in the nucleic acid sequence databases. Alternatively, the single restriction endonuclease restriction digests of pT24 and pT25 could be probed with another known protease gene to determine if there is similarity.

Chapter 5

Conclusion

Three different conjugation techniques, membrane filter mating, plate mating and broth mating, and various parameters were used to associate the protease genes of *P. fluorescens* strains T24 and T25 to the plasmid DNA located in each of these strains. The results of the conjugation experiments and the transformation experiments do not prove nor disprove that the protease genes are located on plasmid DNA.

The heat treatment experiments did not cure *P. fluorescens* strain T24 of its plasmid. However, elevated temperature treatment was able to produce protease negative colonies of *P. fluorescens* strain T25, providing the first line of evidence supporting the plasmid location of the protease genes.

The plasmids pT24 and pT25, from *P. fluorescens* strains T24 and T25, respectively, have been mapped. The pT24 and pT25 plasmids appear to be identicai but they are substantially different from pT20. Plasmid DNA containing a putative cloned protease gene from *P. fluorescens* strain T20 hybridized specifically with a 3.75 kbp *Sal* I fragment and two *Pst* I fragments (sizes 3.4 kbp

and 1.72 kbp) from pT24/pT25 providing a second line of evidence for the plasmid location of the protease genes. In addition, the same probe did not hybridize with digests of genomic DNA from strains T24 and T25 providing the third and final line of evidence supporting the plasmid location of the protease genes.

Additional research is required to conclusively prove whether or not the protease genes are located in the pUT 8 construct and on the plasmid DNAs of the *P. fluorescens* T24 and T25 strains. On the basis of this experiment, and the differences in the restriction maps of pT20 and pT24/pT25, it appears that closely related psychrotrophic *P. fluorescens* strains contain different plasmids.

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Appendix 1

Preparation of various chemical reagents

(1) LB agar with IPTG, X-gal and Amp

950 mL distilled water

10 g Bacto-tryptone

10 g NaCl

5 g Bacto-yeast extract

Adjust pH to 7.4 with 1 M NaOH

Add 15 g agar

Add water to make 1 liter

Autoclave to sterilize

Cool to 50°C, then add 5 mL of 2% X-gal (in DMF), 5 mL of 0.1 M IPTG (in water) and 50 µg/µL ampicillin (in water).

(2) SSC (Sodium chloride/ sodium citrate), 20x

3 M NaCl

0.3 M sodium citrate dihydrate

Adjust the pH to 7.0 with 1 M HCl

Solutions required for the DIG DNA labeling and detection kit protocols.

(3) Buffer 1, Maleic Acid Buffer

0.1 M maleic acid

0.15 M NaCl

Adjust the pH to 7.5(20°C) with solid NaOH

(4) Blocking Stock Solution (10x concentration)

Add powdered blocking reagent, 10% (w/v) in buffer 1. Dissolve the blocking reagent by constantly stirring on a heating block or in a water bath at 65°C, autoclave and store at 4°C.

(5) Buffer 2, Blocking Solution

Prepared by diluting the stock solution 1:10 in maleic acid buffer.

(5) Buffer 3, Detection Buffer

0.1 M Tris-HCl

0.1 M NaCl

50 mM $MgCl_2$

Adjust the pH to 9.5

(6) Standard Hybridization Buffer

5x SSC

N-lauroylsacrosine, 0.1% (w/v)

SDS, 0.02% (w/v)

Blocking solution, 1%

(7) Salt saturated-phenol

1 lb bottle of crystalline phenol

100 mL Tris-HCl, pH 7.4

130 mL water

Heat the mixture at 37°C carefully until the phenol dissolves, then remove the upper aqueous phase.

To the organic phase add the following:

100 mL 2 M Tris-HCl, pH 7.4

25 mL m-cresol (Kodak)

1 mL β-mercaptoethanol

500 mg 8-hydroxyquinoline

Store the aqueous and phenol layers together in a fume hood at room temperature. Use the yellow phenol solution for extraction.

(8) 25:24:1 phenol\chloroform\isoamyl alcohol

(a) Add 0.5 g of 8-hydroxyquinoline to a 2 L glass beaker containing a stir bar.

(b) Gently pour in 500 mL of melted crystals of redistilled phenol (melted in a water bath at 65°C).

(c) Add 500 mL of 50 mM Tris base (unadjusted pH ~ 10.5).

(d) Cover the beaker with aluminum foil and stir for 10 min at low speed with magnetic at RT.

(e) Let phases separate at RT. Gently decant the top (aqueous) phase into a suitable waste receptacle. Remove what cannot be decanted with a 25 mL glass pipette.

(f) Add 500 mL of 50 mM Tris-HCl, pH 8.0. Repeat steps 4 to 6 (ie., two successive equilibrations with 500 mL of 50 mM Tris-HCl, pH 8.0).

(g) Add 250 mL of 50 mM Tris-HCl, pH 8.0, or TE buffer, pH 8.0, and store at 4°C in a brown glass bottle.

(h) For use in DNA purification procedure, mix 25 volumes of phenol with 24 volumes chloroform and 1 volume of isoamyl alcohol.

Appendix 2

Southern Transfer Protocol

The protocol used is that of Ausubel et al. 1995.

An oblong sponge was placed in a glass baking dish, and half-submerged in 0.4 M NaOH. Vertically stacked on top of the sponge was 3 pieces of Whatman 3MM filter paper, wetted with 0.4 M NaOH; the gel, with edges covered with plastic wrap; nylon membrane, flooded with 0.4 M NaOH; 5 pieces of Whatman 3MM paper; paper towel, about 4 cm thick; and a glass plate with a 300 g weight.

The assembled structure was left for approximately o/n. The structure was then disassembled and the membrane recovered. The membrane was rinsed in 2x SSC and placed on a sheet of 3MM Whatman filter paper to dry. The membrane was then stored between sheets of 3MM Whatman filter paper at RT until used for the hybridization experiment.