

INVESTIGATION OF A PLASMID-ASSOCIATED HEAT
STABLE PROTEASE PRODUCED BY PSEUDOMONAS
FLUORESCENS T20

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**INVESTIGATION OF A PLASMID-ASSOCIATED HEAT
STABLE PROTEASE PRODUCED BY *PSEUDOMONAS*
FLUORESCENS T20**

By

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A thesis submitted to the School of Graduate
studies in partial fulfillment of the
requirements for the degree
of Master of Science

Department of Biology
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January, 1990

St. John's

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Abstract

Pseudomonas fluorescens T20, a psychrotroph of milk origin produces an extracellular heat-stable protease which in large quantities causes spoilage of milk and milk products. The protease was purified by affinity column chromatography using carboxy-D-phenylalanine-triethylenetetramine Sepharose 4B. It was shown to have a molecular weight of 44,000.

Two mutant strains of *P. fluorescens* T20 were generated using N-methyl- N'-nitro-N"-nitrosoguanidine. The extracellular extracts of both mutants were shown by immunoprecipitation to be negative for the presence of the heat-stable protease. The intracellular extracts in turn were tested by immunoprecipitation and only one, the Type A mutant strain, showed the presence of cross reacting material. Thus two types of mutants were generated, one which could express the sequence encoding the heat-stable protease but which was unable to export the protease out of the cell and another, which could not express or export the heat-stable protease. The mutant strains were designated Type A and Type B respectively.

The genetic origin of the heat-stable protease was investigated and it was determined that the resident plasmid in *P. fluorescens* T20 carried the gene encoding the heat-stable protease. The plasmid had a molecular weight of 44.06 Kbp. A 3.3 Kbp *Sal* 1 fragment was cloned from the plasmid into the vector, pUC12. The cells transformed with the recombinant showed phenotypic expression of the heat-stable protease. In furtherance of the attempt to find that region of the plasmid encoding the heat-stable protease a restriction endonuclease map of the plasmid was constructed.

Acknowledgements

I wish to express my thanks to my supervisory committee, Dr. T.R. Patel, Dr. P. Hempstead, and Dr. W. Davidson for their example, guidance, and encouragement during the course of this study.

I would also like to thank Dr. E. Barnsley for generously providing the plasmid, pKT230. Also Dr. J. Robinson for his guidance and the provision of the materials for the Western blot procedure.

I wish to thank the School of Graduate Studies and Dr. T. R. Patel for financial assistance over the course of this work.

I also wish to thank Donna Jackman for her assistance during the experimental portion of and also the proof reading of this work.

List of Abbreviations

Amp. = ampicillin

Bac. = bacitracin

BCIP = 5-bromo-4-chloro-3-indolyl phosphate toluidine salt

BSA = bovine serum albumin

CBZ-L-phenylalanine Teta Sepharose 4B = carbobenzoxy-L-phenylalanine
-triethylenetetramine sepharose 4B

Chl. = chloramphenicol

dal. = dalton

DNA = deoxyribonucleic acid

EDTA = ethylenediaminetetraacetic acid

Ery. = erythromycin

Gar-IgG = goat anti-rabbit immunoglobulin G

Kdal = kilodaltons

Kbp = kilobase pair

Kan. = kanamycin

LA = Luria agar

LB = Luria broth

M = molar

Met. = methicillin

mg = milligram

mM = millimolar

mL = milliliter

NBT = *p*-nitroblue tetrazolium chloride

MNNG = N-methyl-n'-nitro-n^o-nitrosoquanidine

nm = nanometers

NP-40 = nonidet P40 (octylphenol-ethylene oxide condensate)

PCA = plate count agar

pDNA = plasmid DNA

Pen. = penicillin

Rif. = rifampin

RNase = ribonuclease

SDS = sodium dodecyl sulfate

SSC = standard saline citrate

Str. = streptomycin

TBS = Tris buffered saline

Tet. = tetracycline

Tris = 2-amino-2(hydroxymethyl)-1,3-propanediol

TSA = trypticase soy agar

TSB = trypticase soy broth

TTBS = tween tris buffered saline

YT = yeast extract tryptone

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Chapter 1

Introduction

1.1 Biological properties of *Pseudomonas fluorescens*

P. fluorescens was first identified in 1889 as *Bacillus fluorescens*. A general definition of its biological properties proposed by Stanier *et al.*, (1966) continues to be applicable today (Doudoroff and Palleroni, 1974). Typically, they are characterized as Gram-negative, rod shaped cells occurring singly. They are approximately 2.0 to 2.8 μm in length by 0.7 to 0.8 μm in width and they are asporogenous, aerobic bacteria that are motile by means of multitrichous polar flagella. Also, as the species name implies, they produce a diffusible fluorescent pigment, phycocyanin. They are chemoorganotrophs, whose metabolism is respiratory, never fermentative. The basic nutritional requirements are quite simple. They can grow in mineral media, supplemented with a single organic compound as the sole source of carbon and energy.

P. fluorescens has been observed to grow at 4°C and at 35°C. Its optimum temperature for growth is 25°C. Subsequently, it has been defined as a psychrotrophic microorganism, that is, one whose optimum temperature of growth lies in the mesophilic range, between 20°C and 45°C, but which can adapt to grow in the psychrophilic range between 0°C and 7°C (Eddy, 1960).

1.2 Properties of extracellular heat-stable proteases

Many genera of psychrotrophic Gram-negative bacteria such as *Acinetobacter*, *Aeromonas*, *Enterobacter*, *Proteus*, and *Pseudomonas* are known to produce

extracellular heat-stable proteases. The best studied are those which are produced by *Pseudomonas*, with *P. fluorescens* as the most common representative. There have been numerous studies on the properties of extracellular proteases produced by psychrotrophic pseudomonads (Richardson, 1981; Yen and Gunsalus, 1982; Patel *et al.*, 1983; and Stepaniak and Fox, 1985). While some biochemical and immunological diversity exists between them (Jackman *et al.*, 1983), general trends are apparent. Most have been classified as neutral metalloproteases (Moriyama, 1974). The optimum pH for activity occurs between pH 7.2 and pH 7.4 (Gebre-Egziabher *et al.*, 1980). As metalloproteases they require divalent cations for activity and stability. Their apoenzymes are generally activated by Ca^{+2} and Mn^{+2} , although high levels of these have proven to be inhibitory (Jackman *et al.*, 1983; Stepaniak *et al.*, 1982).

Temperature optima for *Pseudomonas* proteases occur between 30°C and 45°C (Stepaniak *et al.*, 1982; Patel *et al.*, 1983). Their activities have been observed to decline sharply between 45° and 55°C (Patel *et al.*, 1983). However, they have been shown to retain considerable activity at a temperature range between 60 to 120°C. In the case of *P. fluorescens* AFT 36, it retained about 30% maximum activity at 7°C (Stepaniak *et al.*, 1982).

While many psychrotrophic bacteria are destroyed by pasteurization (Witter, 1961), most produce extracellular proteases which are extremely thermostable and can withstand high-temperature short time exposure (HTST = 72°C/ 15s) and ultra-high temperature (UHT = 138°C/ 2s) treatments (Zall, 1980). Proteases produced by psychrotrophic pseudomonads have been estimated to be quite thermostable since they are 400 and 4000 times more heat resistant than spores of *Bacillus stearothermophilus* and *Clostridium sporogenes*, respectively (Adams *et al.*, 1975). The proteases of thirteen species of pseudomonads isolated from raw milk were shown to retain 55% to 65% of their activity after 77°C/ 17s and 20% to 40% of their original activity after exposure to 140°C/ 5s (Griffiths *et al.*, 1981).

Barach *et al.*(1976) found that the heat-resistance of a Pseudomonad protease was markedly reduced in the absence of calcium. They were led to conclude that the protease was protected at high temperatures by binding calcium. The thermostable properties of the enzyme produced by Pseudomonads are similar to that of thermolysin produced by *B. thermoproteolyticus* with respect to its calcium requirements, lack of sulfhydryl groups, molecular weight, and high content of hydrophobic amino acid residues. Subsequently, it was suggested that these properties allowed the proteases to be flexible when denatured at high temperatures, then to refold quickly and accurately with the formation of calcium salt bridges when the temperature was lowered (Barach and Adams, 1977).

While all isolates produced proteases which retained partial activity after being heated at elevated temperatures between 90°C and 120°C for 10 minutes, the greatest reduction in protease activity occurred when the protease was heated to 50°C for 10 minutes. This inactivation at temperatures just above the optimum temperature for activity, has been referred to as low temperature inactivation, (LTI) (Barach *et al.*, 1978). It has been proposed that LTI occurs when the enzyme undergoes a conformational modification which alters enzyme structure. The altered enzyme then interacts with casein micelles to form an enzyme-micelle complex. Subsequently, the active protease is removed from the milk. However, at temperatures greater than 60°C, hydrophobic interactions are no longer favoured.

Others have suggested that LTI is due to autolysis. This process would occur as a two stage process. First the molecule would undergo a conformational change at 50°C where the molecule opens, resulting in a reversible loss of catalytic activity and susceptibility of the protease to proteolysis by other protease molecules not yet denatured. The second stage involves the association of the denatured enzyme with casein micelles as a result of altered hydrophobic interactions (Barach *et al.*, 1978 and Richardson, 1981).

1.3 Effects of psychrotrophic contamination in milk

Milk is an excellent medium for the growth of a wide variety of microorganisms. The main groups found to constitute the microflora of raw milk include micrococci, streptococci, *Bacillus sp.*, and Gram-positive and Gram-negative rods (Cousin, 1982). In the past, poor cooling was responsible for the development of off flavours and high acid in raw milk, associated with the growth of lactic streptococci and other mesophilic bacteria. Today, however, there is a much higher standard of care taken to ensure that contamination in raw milk is kept to a minimum.

Technological advances in the dairy industry have led to the trend of maintaining raw milk at refrigeration temperatures for extended periods of time. Subsequently, the growth of lactic acid streptococci and other mesophilic bacteria and the spoilage they cause is effectively curbed (Law, 1979). However, this practice of holding raw milk at low temperatures for 4 days or more has resulted in a shift of the major type of microorganisms in milk from mesophilic lactic acid bacteria to psychrotrophic microorganisms, and new problems arise as a result of the ability of these microorganisms to produce a heat-stable protease (Cousin and Marth, 1977 and Stepaniak *et al.*, 1982).

While types of psychrotrophs isolated from raw milk vary, most have been characterized as Gram-negative rods. The most common genera of psychrotrophic organisms isolated from raw milk include *Alcaligenes*, *Arthrobacter*, *Enterobacter*, *Flavobacterium*, and *Pseudomonas*. The most common genus isolated is *Pseudomonas* (Cousin, 1982 and Thomas and Thomas, 1973).

1.4 Effects of psychrotrophic heat-stable proteases on milk, cheese, and other dairy products

It has been widely reported that the heat-stable proteases produced by

psychrotrophic microorganisms lead to gelation and the production of bitter flavours in milk (Adams *et al.*, 1975; Law *et al.*, 1977). Raw milk which had been inoculated with 5×10^6 colony forming units/mL of *P. fluorescens* NCDO 2085 were observed to develop a gel-like texture and a bitter flavour 10 to 14 days after UHT sterilization (140°C/ 3.5 s) and storage at 20°C (Law, 1979). The same result was observed when cell-free culture supernatants of *P. fluorescens* NCDO 2085 and other proteases produced by strains of *P. fluorescens* (Richardson and Newstead, 1979; McKellar, 1981) were added to UHT sterilized milk. This effectively proved that the adverse effects of the milk psychrotrophs were due to an extracellular protease as opposed to one which was cell-bound.

The protease acts by specifically attacking the caseins in milk. It preferentially hydrolyzes β -casein, which results in the accumulation of bitter peptides and subsequently the development of bitterness or off flavours (Grieve and Kitchen, 1985). Most proteases also attack κ -casein, resulting in destabilization of the casein micelles and coagulation of the milk (Law *et al.*, 1977; Gebre-Egziabher *et al.*, 1980). Notably, the whey proteins in milk, such as β -lactoglobulin and α -lactalbumin are insensitive to degradation by psychrotrophic proteases. It has been suggested that this might be due to the globular nature of the whey proteins as opposed to the random non-helical structure of the caseins (Bengtsson *et al.*, 1973, and Law *et al.*, 1977).

Much evidence has been presented which indicates that high counts of psychrotrophs in milk, together with long periods of storage, lead to reduced yields of cheddar cheese (Law *et al.*, 1979). Others have reported that the growth of psychrotrophs in cheese milk reduces rennet coagulation time (Cousin and Marth, 1977). It was established that milks with high counts of psychrotrophs (i.e. > 10 c.f.u./ mL) before pasteurization and pasteurized milk containing culture filtrates of *P. fluorescens* stimulated starter culture growth. The streptococci and lactobacilli used in starter cultures are nutritionally fastidious and require some preformed amino acids. It was proposed that the stimulation of

lactic acid production by the starter culture was due to the activity of the protease on casein which resulted in increased concentrations of peptides and amino acids (Cousin and Marth, 1977).

Other dairy products such as ice cream and butter are not susceptible to the action of psychrotrophic proteases. White and Marshall (1973) found that with the addition of protease from *P. fluorescens* P26 there was no adverse effect on their quality. This is expected since these products contain very little protein. However, it may also be a reflection of the fact that most psychrotrophic proteases have a neutral pH and a temperature optimum between 30°C and 45°C, whereas these dairy products generally have a low pH and are stored below 10°C (Law *et al.*, 1979).

1.5 Genetics of the production of extracellular proteases

While extracellular enzymes of psychrotrophic origin have been studied extensively with respect to their isolation and characterization, the genetic origins and the mechanism underlying their synthesis and regulation has not yet been determined. A model for the regulation of extracellular enzyme synthesis has been proposed which is based on work carried out on Gram-negative microorganisms. It suggests that the regulation of extracellular enzyme synthesis is based on induction, end-product repression, and/or catabolite repression (Harder, 1979). The model assumes that the organism produces a low basal level of extracellular enzyme in the absence of inducer. It suggests that translation occurs at special sites associated with the cytoplasmic membrane and is maintained under specific conditions by a large pool of exoenzyme messenger ribonucleic acid (mRNA) which results from a positive imbalance of transcription over degradation of the messenger.

Indirect evidence has been put forward which suggests that the synthesis of extracellular enzymes occurs on polysomes associated with the cytoplasmic membrane (Both *et al.*, 1972). Direct evidence for this was provided for the

periplasmic enzyme, alkaline phosphatase, of *Escherichia coli*, which was demonstrated to be synthesized on polysomes associated with the membrane (Cancedda and Schlesinger, 1974). Unfortunately, no studies have been reported regarding the mechanism of regulation of the heat-stable protease of *P. fluorescens*. However, by inference, it can be assumed that its regulation follows Harder's (1979) model which is based on work with both Gram-negative and Gram-positive microorganisms. The genera included were *Pseudomonas* (Boethling, 1975; Stinson and Merrick, 1974), and *Vibrio* (Reid *et al.*, 1980).

Direct evidence for the existence of an mRNA pool was provided using the transcriptional inhibitor rifampin-SV. The antibiotic was added to cell suspensions of *P. maltophilia* at various times to a final concentration of 100 $\mu\text{g}/\text{mL}$. When added at zero time, the secretion of the extracellular protease was completely inhibited. However, when it was added to actively secreting cells, in mid-log phase, there was a lag of about 30 minutes in the absence of mRNA synthesis, before the accumulation of protease was inhibited (Boethling, 1975).

1.6 Plasmids in *Pseudomonas*

A plasmid is an extrachromosomal genetic element that is capable of autonomous replication. It most often occurs as circular DNA. Some plasmids can integrate with the host chromosome. In *Pseudomonas* species they can range in size from 5.5 Kb to 300 Kb. In addition to an origin of replication which is essential, plasmids carry genes for a number of other functions. Those which involve plasmid function include genes for the formation of pili or plasmid transfer during conjugation. They have also been shown to include genes which are involved in the host metabolism. The best known of this group are genes which encode antibiotic resistance and they are called R plasmids. Plasmids may in some instances, contain genes that are normally found in the chromosome. This has been found in the *Pseudomonas* plasmid, R68.45. This plasmid has been shown to have a capacity for *in vivo* cloning of selected regions of the bacterial chromosome (Holloway, 1978; Morgan, 1982). On the other hand, genes normally

occurring in plasmids have been found in the bacterial chromosome. Resistant isolates have been found that normally utilize a plasmid determined mechanism of antibiotic resistance, however no R plasmids could be detected. In 30 of 50 gentamycin-resistant clinical isolates of *P. aeruginosa*, gentamycin-modifying enzymes that are usually plasmid mediated could be found. However, R plasmids carrying these genes could be found in only 10 (Jacoby, 1980).

In carbenicillin-resistant clinical isolates from France, the most frequently isolated plasmids carrying the β -lactamase genes were PSE-1 and OXA-2 (Phillippon *et al.*, 1984), while in Great Britain, the most frequently isolated plasmids from such isolates were PSE-4 and OXA-1 (Williams *et al.*, 1984). In the case of plasmid-free strains which nonetheless produced β -lactamase, chromosomally integrated transposons were demonstrated. These results suggest that there is an exchange of resistance genes between plasmids and the *Pseudomonas* chromosome. The ability of bacterial genes to shuttle between chromosome and plasmid might have led to difficulty in distinguishing which genes were of plasmid or of chromosomal origin.

It has been demonstrated that plasmids confer the ability to degrade a diverse group of compounds. They include aliphatic and aromatic hydrocarbons, terpenes, alkaloids, and chlorinated aliphatic and aromatic compounds. A group of plasmids have been found which specify the degradation of *m*- or *p*-xylene, 1,2,4-trimethylbenzene, 3-ethyltoluene, and toluene as well as their corresponding alcohol and acid derivatives. They are known as the TOL plasmids (Williams and Murray, 1974; Reineke and Knackmuss, 1980).

Among the degradative plasmids studied most extensively is the CAM plasmid. This plasmid has been shown to carry a set of genes, many of which are arranged to specify coordinately inducible enzymes, which are involved in the conversion of camphor to isobutyrate (Rheinwald *et al.*, 1973). While both the TOL and the CAM plasmids have been found in *Pseudomonas putida*, other *Pseudomonas* species have been shown to carry degradative plasmids as well. Notably, *P.*

fluorescens has been demonstrated to carry a plasmid, pEG, which confers the ability to degrade styrene (Bestetti *et al.*, 1984).

Species of the genus *Pseudomonas*, particularly the fluorescent species, have been found to be extremely nutritionally versatile (Stanier *et al.*, 1966). Many do not excel in the utilization of sugars, however other organic compounds are readily used by many species. They include hydrocarbons, carbohydrates, aliphatic acids, amines, amides, amino acids, alcohols and aromatic compounds. Some strains have been shown to utilize as many as 100 of 135 substrates (Palleroni, 1981). While not all catabolic pathways are plasmid encoded, they are perceived to be agents of rapid evolutionary change. They can transfer across a wide range of species and genera, and are capable of coming or going as the selection pressure for the genetic elements they encode increases or decreases (Davey and Reaney, 1980). Plasmids have often been implicated in cases where bacteria have adapted to rapid changes in their environment. They include antibiotic resistance, heavy metals, and sequences coding for new catabolic pathways for different or unusual substrates. It is then conceivable that a plasmid might be responsible for the ability of *P. fluorescens* to degrade casein.

The heat-stable protease produced by psychrotrophs such as *P. fluorescens* T20 represents a significant problem for the dairy industry, particularly in view of its adverse effects on milk and other dairy products. While the biochemical and biophysical properties of heat-stable proteases have been studied in some detail, the molecular origins of their production have not been given the same consideration. The objectives of this study were as follows:

- a) To purify the extracellular heat-stable protease of *P. fluorescens* T20.
- b) To find the genetic origin of the protease.
- c) To clone the gene encoding the heat-stable protease and construct a physical map of the region containing the gene.

Chapter 2

Materials and Methods

2.1 Materials

The following chemicals were purchased from Sigma Chemical Company (St. Louis Mo. U.S.A.): Ammonium persulfate, boric acid, bovine serum albumin (BSA), bromphenol blue, calcium chloride, Coomassie brilliant blue R250, ethylenediaminetetraacetic acid (EDTA), Folin-Ciocalteu reagent, glucose, magnesium chloride, sodium acetate, ribonuclease (RNase), sodium acetate, sodium chloride, sodium dodecyl sulphate (SDS), sucrose, trichloroacetic acid (TCA), N-methyl-N'-nitro-N"-nitrosoguanidine (MNNG), and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal).

Reagents such as chloroform, 95% ethanol, isoamylalcohol, isopropyl alcohol, and phenol were obtained from Fisher Scientific Company

The affinity column material, carbobenzozy-L-phenylalanyl-triethylene-tetraaminyl-Sepharose 4B was obtained from Pierce Chemical Company (Rockford Ill.). Material used for gel filtration chromatography, Sephadex G50 and Sephadex G150 and DEAE-cellulose, were obtained from Pharmacia Fine Chemicals (Canada) Inc., Pointe Claire, Quebec.

Reagents used for electrophoresis, agarose, acrylamide and N,N'-methylene-bis-acrylamide, were obtained from Sigma Chemical Company. Noble agar used for immunodiffusion was purchased from Bio-Rad Laboratories (Mississauga, Ont.)

Culture media such as trypticase soy agar (TSA), and trypticase soy broth (TSB) were obtained from Sigma Chemical Company, while tryptone, yeast extract and plate count agar were purchased from Difco Laboratories (Detroit, Mich.).

2.2 Bacterial strains and plasmids used

Bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2 respectively.

Table 2-1: Bacterial Strains Used I

Organism	Relevant phenotype	Source or Reference
<i>A. Escherichia coli</i> JM 83	<i>lacZ</i> - Protease negative	Messing and <i>et al.</i> ,1982
DH1	Protease negative	Low, 1965 Meselson and Yuan 1968 Hanahan, 1983
RR1	Protease negative	Bolivar <i>et al.</i> ,1977 Peacock <i>et al.</i> ,1981

Table 2-2: Bacterial Strains Used II

Organism	Relevant Phenotype	Source or Reference
B. <i>Pseudomonas fluorescens</i>		
T20	Protease positive	Patel <i>et al.</i> , 1983
ATCC 13525	Protease negative	Stanier, 1966

Table 2-3: Plasmids Used:

Plasmid	Phenotype	Source or Reference
pUC 12	amp ^R	Vieira and Messing, 1982
pKT230	kan ^R	Bagdasarian, <i>et al.</i> , 1981
pUT 8	amp ^R Protease positive	This work
pT20	amp ^R , Kan ^R , Protease positive	This work

2.3 Cultivation and maintenance of bacterial strains

P. fluorescens T20 had been previously isolated from raw milk supplied by a local dairy. The raw milk was plated out on plate count agar (PCA) containing 2% skim milk powder. The plates were incubated at 7°C for 8 to 10 days and colonies showing zones of proteolysis were selected and purified by streaking on fresh PCA.

All cultures were started from a single colony and were maintained on the appropriate selective media where, in the case of those strains producing the heat-stable protease, milk powder was included in the media. On the other hand, to ensure that plasmids were maintained, the media included the appropriate antibiotics. The media used were as follows: trypticase soy agar (TSA) plates and broth (TSB), Luria agar (LA) plates and broth (LB) (Miller, 1972), yeast extract tryptone (YT) plates (Miller, 1972). When required, skim milk powder was added to a final concentration of 2% and antibiotics such as ampicillin, kanamycin, and tetracycline were added to a final concentration of 20 µg/mL, 50 µg/mL, and 25 µg/mL respectively. Pseudomonads were grown in trypticase soy media while the *E. coli* strains were maintained on both the LA and YT media.

2.4 Generation of a protease deficient mutant

Trypticase soy broth (9 mL) was inoculated with preinoculum (1 mL), which had been grown overnight to mid-log phase. N-methyl-N'-nitro-N"- nitrosoguanidine (MNNG), was added at a concentration of 1 µg/mL to 30 µg/mL. The cultures were grown overnight at 25°C. Those cultures where MNNG just permitted growth were selected. The cells were pelleted by centrifugation at 5,000 g for 10 minutes at 25°C. The pellets were washed twice with Tris-HCl buffer (20 mM pH 7.5), resuspended in the original culture volume of Tris-HCl buffer and a series of dilutions ranging from 1:10 to 1:1,000,000, were prepared and plated out in 100 µL aliquots on selective media (TSA and 2% milk powder). The plates were incubated at 25°C and checked at 24-hour intervals over a period of 3 days for

protease-deficient strains, as indicated by the lack of a zone of proteolysis surrounding the colony.

2.5 Preparation of the bacterial extracts for the heat-stable enzyme assay

Starting cultures were grown overnight in 10 mL of liquid medium overnight. The optimum temperatures were 25°C for *P. fluorescens* T20 and the ATCC culture and 37°C for the *E. coli* strains. These were used to inoculate experimental cultures (200 mL in 1000 mL flasks) of TSB or LB supplemented with 2% milk powder. Cells were grown at their optimum temperatures for 2 to 3 days in a shaker (Psychrotherm, New Brunswick Scientific Co., New Brunswick, N.J.). Cells and any unhydrolyzed milk were removed by centrifugation at 12,000 *g* for 15 minutes at 4°C. The clear supernatant, containing the heat-stable enzyme, was concentrated by ultrafiltration and dialyzed for 12 hours at 4°C against 20 mM Tris-HCl buffer (pH 7.2). The dialyzed extract was the source of crude heat-stable protease.

Intracellular extracts were prepared as follows: The cells were harvested by centrifugation at 12,000 *g* for 15 minutes at 4°C. The pellet was resuspended in 25 mL of cold 100 mM Tris-HCl (pH 8.0) and centrifuged as before. The washing was repeated and the final pellet was resuspended in breakage buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl₂), which was one fiftieth of the original culture volume. Cells were broken by sonication with a Braun-Sonic Model# 2000 using a fine tip probe. There were 5 sonications, each of 20 seconds, interspersed with a period of one minute of cooling on ice.

2.6 Heat-stable protease assay

The protease activity in enzyme preparations was estimated by a modification of the method of Hull (1947) as described by Patel *et al.*, (1983). Soluble casein was used as the substrate (BDH Chemicals Ltd., Dartmouth, Nova Scotia). It was

extensively dialyzed against 100 mM Tris buffer (pH 7.5) to remove any contaminating free amino acids to ensure optimum protease activity. The reaction mixture was composed of 1.3 mL Tris-HCl (100 mM, pH 7.5), 0.2 to 0.4 mg protein in a total volume of 0.2 mL, and 0.5 mL of casein (1%). The reaction mixture was incubated at 25°C for 20 minutes in a temperature-regulated water bath. The reaction was stopped by the addition of 1 mL of 5 % trichloroacetic acid (TCA). The precipitated proteins were removed by centrifugation at 12,000 *g* for 10 minutes at 4°C. The acid-soluble free tyrosine and tryptophan were determined by absorbance measurements at 280 nm. Appropriate enzyme and substrate controls were included. One enzyme unit was defined as the amount of extract that produced 1 μ mol of tyrosine/min/mL under assay conditions. Protein was determined according to the method described by Lowry *et al.*(1951).

2.7 Purification of the protease

All procedures were carried out at 0 to 4°C. Crude extract (400 mL) was concentrated by ultrafiltration to 40 mL using a UM-10 membrane (Amicon Corp., Oakville, Ontario, Canada). The concentrated protease was dialyzed against a sodium acetate buffer (25 mM sodium acetate pH 5.8, 100 mM NaCl, 10 mM CaCl₂) and was applied to an affinity column with a void volume of 10 mL, consisting of carbobenzoxy-D-phenylalanine-triethylenetetramine Sepharose-4B (Pierce Chemical Co., Rockford, Ill.). The unbound protein was eluted with about 200 mL of the acetate buffer. The bound protein was eluted with 200 mL of a high salt buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, and 10 mM CaCl₂). The fractions containing the active protease were pooled and concentrated to 5 mL using ultrafiltration.

2.8 Estimation of the molecular weight of the heat-stable protease

The protease was run through a column (2 x 45 cm) packed with Sephadex G 150 which had been calibrated with the following proteins: Ferritin (440,000), catalase (210,000), aldolase (158,000), alcohol dehydrogenase (141,000), bovine

serum albumin (64,000), ovalbumin (43,000), chymotrypsin (25,000), and lysozyme (14,400). A standard curve was obtained by plotting the log of the molecular weight against the retention coefficient (K_{av}) of the various proteins. All proteins were eluted from the column with Tris-HCl buffer (20 mM, pH 7.5). The absorption at 280 nm was measured to determine the concentration of the protein in the fractions and the enzyme activity was determined by a modification of Hull's method as described by Patel *et al.* (1983).

2.9 Polyacrylamide gel electrophoresis

2.9.1. Disc gel method

Protein homogeneity was examined by disc gel electrophoresis as described by Weber and Osborn (1969). Electrophoresis was carried out on 7.5% polyacrylamide gels (refer to Appendix I) with 0.1% SDS at pH 9.5. Between 50 and 100 μ g of protein in a volume of 100 μ l was loaded on the gel in 200 μ l of sample buffer (refer to Appendix I). The sample was heated at 90° C for 2 to 3 minutes, 5 μ l of 0.1% bromophenol blue was added and the samples were loaded onto the gel. The gels were run at 1 mAmp/tube initially until the proteins had run into the running gel, after which time they were run at 2 mAmp/tube until the tracking dye was approximately 1 cm from the bottom. The protein bands were fixed overnight in 50% TCA, then stained in 0.1% Coomassie brilliant blue in 50% TCA for 2 hours. To destain, the gels were soaked in 7% acetic acid.

2.9.2. Slab gel method

The 12.5% gel was prepared as indicated in Appendix II and poured between two glass plates to a level of 12 cm, overlaid with butanol, and allowed to set at room temperature for 30 minutes. The gel was then rinsed thoroughly with running buffer. Excess liquid was removed with filter paper. Next the stacking gel was layered on top of the running gel and it was allowed to set for about 30 minutes at room temperature. The protein samples were prepared as described for the disc gel method, however only 10 to 40 μg of protein were added to each of the wells. The gel was run at 75 volts for approximately 30 minutes giving the protein time to move into the running gel, after which time the voltage was increased to 150 volts. The proteins were allowed to run for about 3 hours or until the tracking dye (0.1% Bromophenol blue), was about 1 cm from the bottom of the gel. The protein was stained with 0.1% coomassie brilliant blue in 10% acetic acid and 25% isopropanol. After which, the gels were destained with 10% acetic acid : 25% isopropanol until the background had been lifted from the gel.

2.10 Western blotting: Immunodetection of proteins

Proteins separated on a 12.5% SDS-polyacrylamide gel as described in section 2.9 were electroblotted onto a nitrocellulose membrane (0.45 micron, Bio-Rad Laboratories, Mississauga, Ont.). After the gel was equilibrated in transfer buffer (250 mM Tris, 1.92 M glycine, and 20% methanol), the protein bands were electroblotted onto nitrocellulose paper overnight at 200 mA in transfer buffer (Towbin *et al.*, 1979). Preparation of the nitrocellulose for detection with polyclonal antibodies was carried out using a modification of the method described by Burnette (1981) and is outlined below. The nitrocellulose was washed for 5 to 6 hours in blocking buffer (20 mM Tris -HCl, pH 7.5, 500 mM NaCl, and 2% BSA). Every hour the buffer was replaced with fresh buffer.

Purified IgG raised against the purified protein (section 2.11) was diluted 1:500 in wash buffer (50 mM Tris-HCl, pH 7.5 0.15 M NaCl, 0.05% NP40 (Sigma

Chemical Co.), 0.1% SDS, 20 mM NaI, and 2% BSA). The filter was then transferred to this buffer and was incubated at room temperature, overnight, with shaking. It was then washed in washing buffer for 5 to 6 hours at room temperature, replacing the buffer with fresh buffer every hour. The filter was then washed with 100 mL of Tween Tris buffered saline (TTBS) (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.05% Tween 20(Sigma Chemical Co.)), replacing the buffer four times, at 20 minute intervals.

The secondary antibody, goat anti-rabbit IgG (GAR-IgG) alkaline phosphatase conjugate (Bio-Rad Laboratories) was diluted 1:3,000 in TTBS and 2% BSA. The filter was then incubated in this buffer for one hour at room temperature with shaking. Following this, the filter was washed four times at 20 minute intervals with 100 mL of TTBS. A final wash with 100 mL of Tris buffered saline (TBS) was carried out for 20 minutes.

Colour development of the bound secondary antibody was accomplished using a mixture composed of 1 mL of 3.0 % *p*-nitroblue tetrazolium chloride (NBT) (Bio-Rad Laboratories) and 1 mL of 1.5 % 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (BCIP) (Bio-Rad Laboratories) in 100 mL of carbonate buffer (0.1 mM NaHCO₃, 1 mM MgCl₂ pH 9.8). Color development was allowed to proceed for 5 to 10 minutes, and was stopped by immersion of the filter paper in distilled water.

2.11 Antiserum production and Ouchterlony analysis

Antiserum to purified protease T25 had been previously prepared using randomly bred New Zealand white rabbits. Injections of purified protease (100 µg) in Freund adjuvant (Difco Laboratories, Detroit, Mich.) was administered subcutaneously at 2-week intervals (Jackman *et al.* 1983). The immunoglobulin G (IgG) was purified from whole serum according to the sodium sulfate precipitation method of Kekwick (1940) and the DEAE-cellulose chromatography method of Levy and Sober (1960).

Ouchterlony analysis was performed as described by Stollar and Levine (1963) in 1.5% Noble agar (Difco Laboratories, Detroit, Mich.). The Noble agar was prepared using Tris-barbitol buffer (1%) at pH 8.6 containing 0.02% sodium azide.

2.12 Isolation of plasmid DNA

2.12.1 Small scale isolation of plasmid DNA

A single colony was inoculated into 10 mL of TSB and grown for 12 to 16 hours in a gyratory shaker. The cells were pelleted at 5,000 g for 10 minutes at 4°C. The pellet was resuspended in 600 μ L of cold sucrose-Tris (1 mM sucrose, 50 mM Tris-HCl, pH 7.4). To this, 1.8 mL of SDS-NaOH-EDTA (1 % SDS, 200 mM NaOH, 5 mM EDTA, pH 12.45) was added and the solution was allowed to stand at room temperature for 20 minutes before addition of 2 mL of 2 M sodium acetate (pH 5.1). The mixture was allowed to stand on ice for 1 hour. Protein-SDS complexes and denatured DNA were removed by centrifugation at 5,000 g for 20 minutes at 4°C. DNA was precipitated by the addition of 4.5 mL of isopropyl alcohol and then incubated at -20°C for 1 hour. The DNA was pelleted at 5,000 g for 30 minutes at 4°C. The supernatant was decanted and the pellet was dried under vacuum. The pellet was resuspended in 500 μ L of Tris-EDTA (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). To this 2.5 μ L of RNase (10 mg/mL of distilled water) was added and the suspension was allowed to stand on ice for 30 minutes. This was followed by the addition of 50 μ L of 1 M NaCl and 500 μ L of phenol:chloroform:isoamylalcohol (50:49:1). The suspension was held at room temperature for 5 minutes, followed by a 4 minute period of centrifugation at full speed in an Eppendorf microfuge. The upper aqueous layer was transferred to a fresh 1.5 mL microfuge tube. To this 50 μ L of 2 M sodium acetate and 1 mL of 95% ethanol were added to precipitate the plasmid DNA. The mixture was incubated at -20°C for at least one hour. DNA was pelleted as before, by centrifugation at 4°C. The supernatant was decanted and the pellet was dried under vacuum. The pellet was resuspended in 200 μ L of Tris-EDTA and stored at -20°C.

2.12.2 Large scale isolation of plasmid DNA

The extraction and purification of plasmid DNA was achieved by a modification of the alkaline lysis procedure described by Maniatis *et al.* (1982). Plasmid DNA in *E. coli* strains was amplified by growing in LB (250 mL) supplemented with chloramphenicol to a final concentration of 200 µg/mL as described by Clewell (1972). *Pseudomonas* cultures were not amplified.

After inoculation, cultures were incubated for 12 to 16 hours at the optimum temperature. Cells were pelleted at 5,000 *g* for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). After standing at room temperature for 20 minutes, 10 mL of freshly made Solution II (200 mM NaOH, 1% SDS, pH 12.45) was added. The suspension was mixed by gentle inversion and held on ice for 20 minutes. Following this, 7.5 mL of Solution III (3 M potassium acetate, pH 4.8), was added. The suspension was mixed by gentle inversion and allowed to stand on ice for 20 minutes. Cellular debris, protein-SDS complexes, and denatured DNA were removed by centrifugation at 15,000 *g* for 1 hour at 4°C. Plasmid DNA was precipitated by the addition of 0.6 volumes of isopropyl alcohol to the supernatant. This suspension was allowed to stand at room temperature for 30 minutes. The plasmid DNA was recovered by centrifugation at 8,000 *g* for 1 hour at room temperature. The DNA pellet was dried under vacuum and resuspended in a minimum volume of Tris-EDTA buffer (10 mM Tris, pH 8.0, 1 mM EDTA). Further purification of covalently closed circular DNA was achieved by buoyant density centrifugation in CsCl gradients containing ethidium bromide (Maniatis *et al.*, 1982).

2.13 Transformation of bacterial strains

2.13.1 Transformation of *Escherichia coli*

E. coli cells were prepared for transformation according to the method of Cohen

et al. (1972) as modified by Dillon *et al.* (1985). An overnight broth culture was diluted 1:100 in 50 mL LB and grown to mid-log phase ($A_{600}=0.5$) in a 37°C shaking water bath for about 90 to 120 minutes. The cells were chilled in an ice bath for 15 minutes and then pelleted by centrifugation at 7,000 *g* for 10 minutes at 4°C. The supernatant was resuspended in 50 mL of cold 0.1 M MgCl₂, then pelleted as before and resuspended in 25 mL of cold 0.1 M CaCl₂ and chilled for 20 minutes in an ice bath. The cells were pelleted as before and resuspended in 2.5 mL cold 0.1 M CaCl₂. To 200 µl of competent *E. coli* cells, 100 µl of the DNA to be used was added at a concentration of 1 to 5 µg/mL. The cells were then kept on ice for 10 minutes, before being diluted 1:10 into pre-warmed Luria broth and incubated with moderate shaking at 37°C for 90 to 120 minutes. They were then plated out onto selective media (refer to section 2.3) and incubated overnight at 37°C.

2.13.2 Transformation of *Pseudomonas* spp.

Pseudomonas strains were prepared for transformation according to the method of Potter and Loutit (1982), as modified by Dillon *et al.* (1985). An overnight culture of *Pseudomonas* in BHI supplemented with 0.8 g/L of KNO₃ was diluted 1:10 to a final volume of 10 mL in broth and incubated with gentle shaking for 4 hours at the optimum temperature in a 30 mL flask in a gyratory shaker. Cells were harvested by centrifugation at 8,000 *g* for 10 minutes at 4°C. The cell pellet was then resuspended in 5 mL of chilled 0.1 M MgCl₂ and centrifuged as before. The pelleted cells were resuspended in 5.0 mL of chilled 0.15 M MgCl₂ and then set in an ice bath for 20 minutes. The cells were then pelleted out as before and resuspended in 1 mL of chilled 0.15 M MgCl₂.

To 200 µl of competent cells, 100 µl of DNA to be transformed was added at a concentration of 1 to 3 µg/mL. The transformation mixture was then set on ice for 30 to 60 minutes. The mixture was heat-pulsed for 2 minutes at 42°C, then stood at room temperature for 5 minutes, before being diluted 1:10 to a final volume of 3 mL in pre-warmed BHI broth and incubated at optimum temperature

for 90 minutes. The transformed cells were then plated out on selective media and incubated at optimum temperature overnight.

2.14 Endonuclease digestion and ligation of DNA

Restriction endonuclease digestion of plasmid DNA was carried out using the restriction endonucleases *Sal* I, *Bam* HI, *Pst* I, *Eco* RI, *XBa* I, *Hind* III, and *Sac* I which were obtained from Boeringer-Mannheim (Dorval Que.). The reactions were carried out in 20 μ l volumes consisting of 3 μ l water, 3 μ l BSA (1 mg/mL), 3 μ l 10x core buffer (Boeringer-Mannheim), 10 μ l plasmid DNA (5 to 10 μ g) and 1 μ l of restriction endonuclease (1 to 2 units). The reactions were incubated for 12 to 16 hours at 37°C.

Ligation of DNA fragments was carried out using T4-DNA Ligase which was obtained from Gibco/BRL Life Technologies (Dorval, Que). The reaction was carried out in a 20 μ L volume. The vector DNA and the plasmid DNA to be cloned were digested as described above except that 0.5 μ g DNA was used. After digestion was complete, two volumes of ice-cold ethanol were added to the digests to precipitate the DNA fragments over a 2 hour incubation period at -20°C. The precipitated DNA was pelleted out of solution by centrifugation at full speed in an Eppendorf centrifuge for 15 minutes. The supernatant was removed and the pellets were dried in a vacuum. The DNA fragments were then resuspended in ligation buffer (20 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 10 mM dithiothreitol, 0.6 mM ATP (Sigma Chemical Co.), and 0.1 units of T4-DNA ligase was added. The reaction mixture was then incubated at 12°C for 12 to 16 hours in a refrigerated water bath.

2.15 Digestion of restriction endonuclease digested DNA with alkaline phosphatase

The 5' phosphate group was removed from the linearized plasmid DNA with calf-intestinal phosphatase (Boehring-Mannheim). The reactions were carried

out according to the recommendations of the manufacturer. The dephosphorylated DNA was heated at 70°C for 20 minutes to destroy calf-intestinal phosphatase activity and was extracted with phenol as described by Maniatis *et al.* (1982).

2.16 Agarose gel electrophoresis

Horizontal agarose gel electrophoresis was carried out in 0.5% or 0.8% agarose Type 111 (Bio-Rad) in Tris-Borate-EDTA buffer (Maniatis *et al.*, 1982). Samples were loaded in a 30 μ L volume containing 1 μ g DNA in a 1:5 dilution with a 50:50 solution of TBE (10 X) and glycerol. The gels were run at 50 to 60 volts for 3 hours, horizontally at room temperature, stained in ethidium bromide (0.4 μ g/mL), and photographed with a red filter using a polaroid camera with Kodak 660 film. The *Hind* 111 restriction endonuclease digests of bacteriophage lambda DNA (Boehringer-Mannheim) were used to prepare a standard curve in which the distances migrated by the restriction fragments of the bacteriophage lambda DNA were plotted against the logarithm of their size in kilobase pairs (Kb). The standard curve was used to determine the molecular weight of uncharacterized restriction fragments using their distances of migration.

The digestion sites of a series of restriction endonucleases, determined from analysis of the sizes of the fragments generated by single and multiple digestion of the plasmid DNA, were confirmed using the MAPC/MAPL restriction mapping programs in Fortran available on VMS in the ICR sequence program package. The package was compiled by Holly Cael and Peter Young of the Fox Chase Cancer Center, Philadelphia; and adapted for use at Memorial University of Newfoundland by Donna Green of Computer Services, Faculty of Medicine, Memorial University of Newfoundland.

2.17 ³²P-labelling of plasmid DNA

Plasmid DNA was radioactively labelled by nick translation, using a kit

purchased from Amersham Inc. according to the manufacturers instructions. Unincorporated nucleotides were removed by column chromatography on Sephadex G 50 using 100mM Tris-HCl pH 8.0, 1mM EDTA pH 7.8. DNA fractions which contained a minimum of 10^8 cpm were pooled and used for molecular hybridization.

2.18 Molecular hybridization:

DNA fragments were immobilized on nitrocellulose filters according to the method of Southern (1975). First the DNA was denatured *in situ*, by immersion in 250 mL of solution 1 (1.5 M NaCl, 0.5 M NaOH) for 30 minutes at room temperature with shaking. This was replaced with 250 mL of solution 2 (1.5 M NaCl, 0.5 M Tris pH 7.2). The gel was washed with this solution twice each time for a period of 20 minutes. The DNA fragments were transferred to nitrocellulose filters in 8x standard saline citrate (SSC), (Appendix III). Non-specific binding sites on the nitrocellulose filters were saturated with herring sperm DNA (50 $\mu\text{g}/\text{mL}$) in 10 mL of a prehybridization solution (5x Denhardt's (Denhardt, 1966) (Appendix III), 6x SSC, 50% formamide, water, and 0.1% SDS) at 45°C for 12 to 16 hours (Thomas, 1980). The prehybridization solution was replaced with one whose composition was identical but which contained the radioactive probe DNA. The radioactive probe was prepared for hybridization by incubation in a boiling water bath for 10 minutes, which converted it to the single strand form. It was then immediately cooled in an ice bath ensuring that the probe would not recircularize. Hybridization was then carried out for 12 to 16 hours at 45°C.

The filters were prepared for autoradiography by two washes each of 15 minutes with 250 mL of 2x SSC at room temperature. The filters were then washed once with 500 mL of 0.1% SDS for 30 minutes at 50°C. SDS blocked the adsorption of single strand DNA, thus facilitating the removal of unbound probe. The filters were blotted dry, placed on 3MM Whatman filter paper, and covered with saran wrap. The filters were exposed to Kodak X-ray film XRP-1 for 12 to 16 hours at -70°C.

Chapter 3

Results

3.1 Extraction and purification of the heat-stable protease produced by *Pseudomonas fluorescens* T20

The steps used for purification and extraction of the heat-stable protease from the crude extract of *P. fluorescens* T20 are outlined in Table 3.1. As indicated, about 20 % of the enzyme was recovered after purification by affinity chromatography. Since the crude extract had a concentration of 200 mg in 500 mL and after affinity column chromatography the concentration of the purified protein was 6.0 mg per mL, the pure enzyme represents about 3.0 % of the total amount of protein loaded onto the column. The purified protein had a concentration of 0.40 mg per mL. After ultrafiltration, it had a concentration of 0.6 mg per mL.

The pure T20 protease was separated from the crude extract by affinity chromatography using CBZ-L-phenylalanine TETA Sepharose 4B. Two protein peaks were eluted as is shown in Figure 3.1. The first major protein peak contained mostly unbound protein and gave no enzymatic activity when assayed using Hull's method. A new elution buffer, which increased the salt concentration from 100 mM to 500 mM and also increased the pH from 5.8 to 8.0 was applied to the column and the bound proteins were then eluted in one peak. The fractions which comprised this peak were then pooled and used as the source of the

enzyme. The purified protease was analysed on a 7.5% SDS-polyacrylamide gel pH 9.5. As shown in Figure 3.2, a single band was observed, indicating that *P. fluorescens* T20 produces only one heat-stable protease and that affinity column chromatography yields substantially pure protein.

**Figure 3.1 Elution profile of purified T20 protease on
CBZ-L-phenylalanine TETA Sepharose 4B**

A column (0.8 x 20 cm) was packed with carboxy-D-triethylenetetramine sepharose-4B affinity column chromatography material. The starting buffer consisted of 25 mM sodium acetate pH 5.8, 100 mM sodium chloride, and 10 mM calcium chloride. The position of the → indicates where the elution buffer consisting of 100 mM Tris-HCl pH 8.0, 500 mM sodium chloride, and 10 mM calcium chloride, replaced the starting buffer.

—●— protein
—■— activity

Figure 3-1: Elution profile of purified T20 protease on CBZ-L-phenylalanine TETA Sepharose 4B

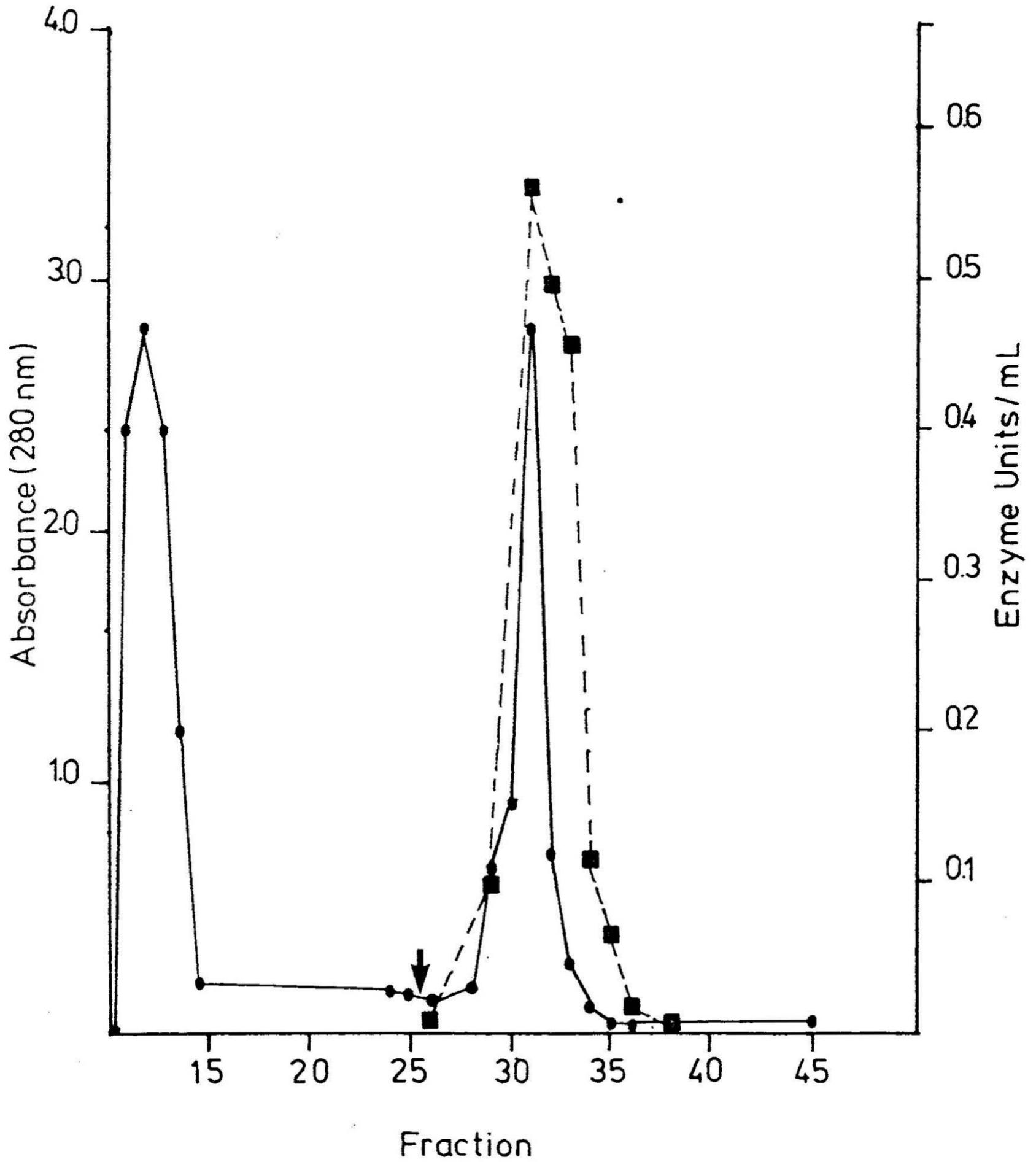


Figure 3.2 Homogeneity of purified T20 protease on 7.5% SDS-polyacrylamide gels

Denatured purified T20 protease (80ug) was layered on to a 7.5% SDS-polyacrylamide gel. Bromophenol blue (0.1%) was used as the tracking dye. The electrophoresis was completed as described in section 2.9.1.

The arrow indicates the position of the marker dye when electrophoresis was stopped.

Figure 3-2: Homogeneity of purified T20 protease on 7.5% SDS-polyacrylamide gels

Step	Volume (ml)	Total protein (mg)	Enzyme units ^a	Enzyme sp. act. ^b	% Enzyme unit recovery
Crude extract	500	14.9	0.38	100	
Cohen's by ultra-filtration	50	9.7	1.5	40.1	
Affinity column	15	4.8	2.5	100	

T 20



^aOne unit of enzyme is defined as the amount of enzyme that will liberate 1 μmole of tyrosine equivalent per minute under standard assay conditions.

^bSp. act. is defined as the amount of enzyme per mg of protein.

produces 1 μmole of tyrosine equivalent per minute under standard assay conditions.

Table 3-1: Purification of the heat-stable protease produced by *Pseudomonas fluorescens* T20

Step	Volume (mL)	Total protein (mg)	Enzyme units ^a	Enzyme sp. act. ^b	% Enzyme unit recovery
Crude extract	500	200	74.0	0.38	100
Concen'n by ultra-filtration	50	20	29.7	1.5	40.1
Affinity column	15	6.0	14.8	2.5	20.0

^aOne unit of enzyme required to produce 1 μ mole of tyrosine equivalent per minute under standard assay conditions.

^bUnits per mg of protein.

3.2 Estimation of the molecular weight of the purified T20 protease

The molecular weight of the purified T20 protease was determined by gel filtration on a Sephadex G150 column which had been calibrated with proteins of known molecular weight (refer to section 2.8). Their retention coefficients (K_{av}) were determined and are shown in Table 3.2. A curve of the logarithm of the molecular weight versus the retention coefficient, (K_{av}) for these standards was used to estimate the molecular weight of the T20 protease. As shown in Figure 3.3, the pure T20 protease had an elution volume (V_E), close to that of ovalbumin. Calculation of the retention coefficient showed it to be 51.4 hence the protein had a molecular weight of approximately 44,000.

Figure 3.3 Calibration curve of the logarithm of the molecular weight of standard proteins versus the retention coefficient (K_{av}) on Sephadex G 150

A column (2x 45 cm) was packed with Sephadex G150. The column was calibrated with those standards indicated in the figure and their retention coefficients were calculated using the following formula:

$$K_{av} = \frac{V_E - V_0}{V_T - V_0}$$

where:

V_E = Volume to protein peak
 V_0 = Elution volume of blue dextran
 V_T = Volume of the column

Figure 3-3: Calibration curve of the logarithm of the molecular weight of standard proteins versus the retention coefficient K_{av} on Sephadex G150

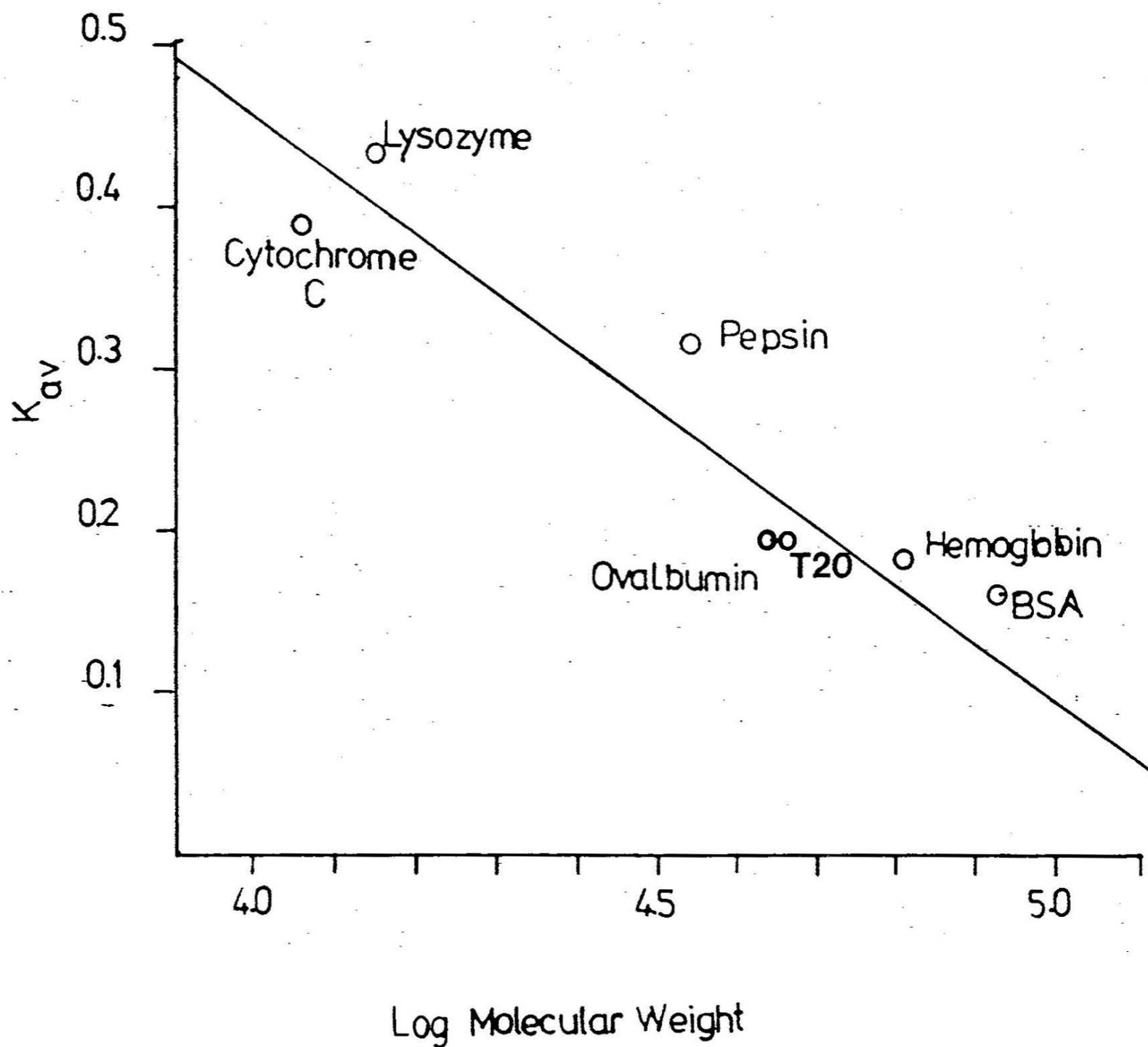


Table 3-2: Retention coefficients of standard proteins eluted on Sephadex G150 and the pure *P. fluorescens* T20 protease

Protein	Molecular weight	Elution volume (mL)	Retention coefficient
blue dextran	200,000	33.6	
bovine serum albumin	68,000	48.3	0.162
ovalbumin	44,000	51.6	0.198
hemoglobin	64,000	50.4	0.185
lysosyme	14,400	73.1	0.434
cytochrome C	11,700	69.3	0.392
pepsin	35,000	62.4	0.317
T20 protease	44,000	51.4	0.197

3.3.0 Genetics of protease production

3.3.1 Construction of a protease-deficient mutant

Mutants of *P. fluorescens* T20 which were unable to either express or export the extracellular protease were generated as described in section 2.4. The concentrations which just permitted growth were those containing less than or equal to $3 \mu\text{g mL}^{-1}$ of MNNG. Good and moderate growth was observed in 1 and $2 \mu\text{g mL}^{-1}$ MNNG respectively, while only scant growth appeared in the presence of $3 \mu\text{g mL}^{-1}$ MNNG. Serial dilutions from 10^{-1} to 10^{-7} were prepared from that concentration which just permitted growth ($3 \mu\text{g mL}^{-1}$) and 1 mL aliquots were plated out on to 2 % milk powder/TSA plates. Mutants were identified by the absence of a zone of proteolysis surrounding the colony, indicating an inability to produce the heat-stable protease. Four colonies were isolated which did not produce zones of proteolysis. They were isolated from a 1 mL aliquot derived from a culture containing 73×10^4 c.f.u. mL^{-1} . Both intracellular and extracellular extracts of the mutant strains were assayed for protease activity. None of the mutants showed any protease activity in either the intracellular or extracellular extracts.

3.3.2 Immunoprecipitation of T25 antisera against mutant extracts

Antiserum raised against the T25 protease was purified to IgG and was used to determine whether the intracellular extracts contained cross reacting but inactive protein. In fact, as illustrated in Figure 3.4, two types of mutants were generated. Two of the strains, T20A mutants, showed immunoprecipitation of the intracellular extract alone, while the remaining two mutant strains, T20B showed no precipitin reaction in either the intracellular or in the extracellular extracts. In Figure 3.4, for purposes of clarity one strain of each mutant type is shown.

3.3.3 Isolation of a plasmid from *P. fluorescens* T20 and its mutant strains

A plasmid was isolated from the *P. fluorescens* T20 strain using the large scale alkali-lysis method described in section 2.11.2. Figure 3.5 shows the result of electrophoresis of the plasmid DNA on a 0.5% agarose gel. The mutant strains, T20A and T20B, were also examined for the presence of a plasmid of the same molecular weight. Only T20A was shown to contain a plasmid which migrated the same distance on the gel. The T20A mutant was also examined for the presence of the heat-stable protease in both the intracellular and the extracellular extracts by immunoprecipitation with the purified IgG (refer to section 2.10). Only the intracellular extracts showed the presence of a heat-stable protease. T20B strains did not carry a plasmid and when tested for the presence of the heat-stable protease they did not yield cross reacting material in either the intracellular or the extracellular extracts.

3.3.4 Antibiotic resistance spectra of the mutant versus wild type strains of *P. fluorescens* T20

While many *Pseudomonas* species are known to be intrinsically resistant to many antibiotics due to the structure of the cell wall, a number of *Pseudomonas* species have been shown to carry R plasmids or resistance plasmids which mediate resistance to a great number of antibiotics. Since the T20B mutant strain, had apparently been cured of its plasmid, it was necessary to determine if its antibiotic resistance spectra had been altered. Table 3.3 shows the pattern of antibiotic resistance in the wild-type and protease deficient mutant T20 strains. The patterns of antibiotic resistance remained the same for both the wild type and the mutant strains, except that both mutant types were shown to be sensitive to chloramphenicol 10 μ g/mL, while the wild type was not.

Figure 3.4 Immunoprecipitation of T20 protease in cell extracts of protease deficient mutants

The Ouchterlony plates were prepared using 1.5% noble agar in 1% Tris-barbitol buffer pH 8.6. The plates were incubated at 24°C in a humidity chamber for 48 hours. Intracellular and extracellular extracts were prepared as described in section 2.5.

- A = pUT8 intracellular extract
- B = T20 crude extract
- C = T20 purified extract
- D = Pseudomonas fluorescens Type A mutant extracellular extract
- E = P. fluorescens Type A mutant intracellular extract
- F = P. fluorescens Type B mutant extracellular extract
- G = P. fluorescens T25 IgG

**Figure 3-4: Immunoprecipitation of T20 protease
in cell extracts of protease deficient mutants**

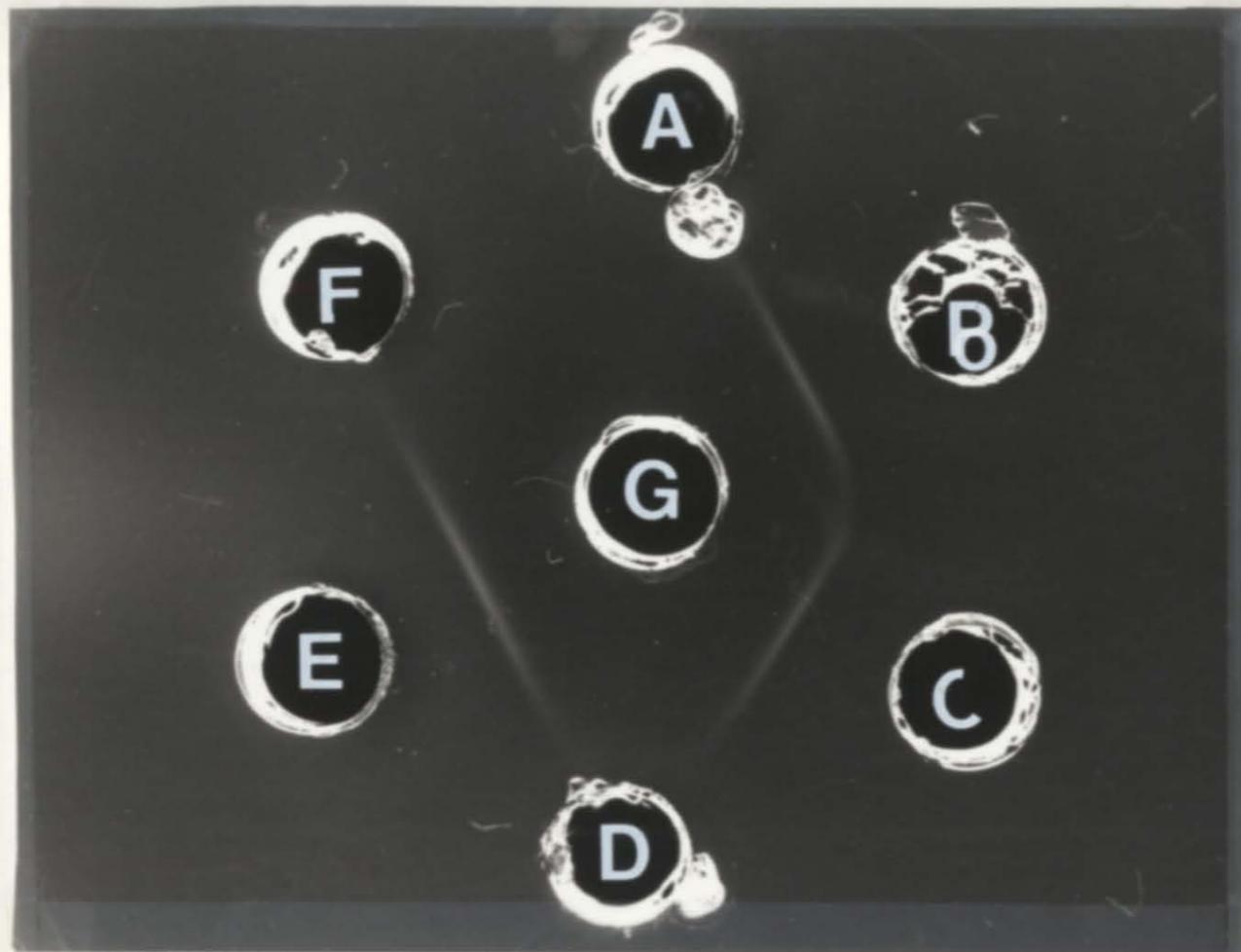


Figure 3.5 Visualization of plasmid DNA occurring in *P. fluorescens* T20 and its mutant strains

Plasmid DNA was prepared as described in sections 2.12.2. Electrophoresis of the isolated plasmids was carried out on a 0.5% agarose gel as described in section 2.16.

Lane 1 = P. Fluorescens T20
Lane 2 = P. fluorescens Type A mutant
Lane 3 = P. fluorescens Type B mutant

Figure 3-5: Visualization of plasmid DNA occurring in *P. fluorescens* and its mutant strains

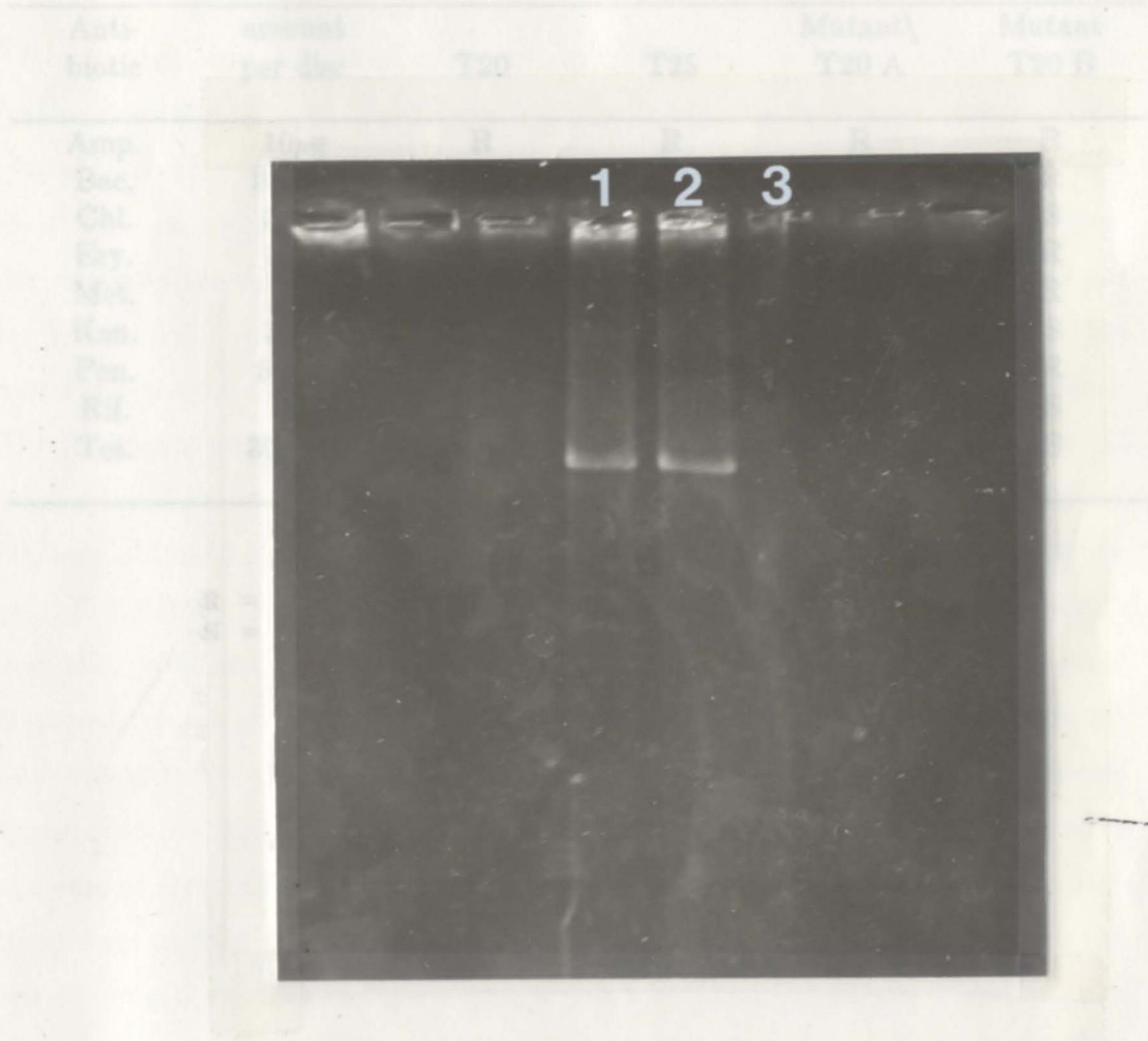


Table 3-3: Antibiotic resistance spectra for the wild type and the protease deficient *P. fluorescens* T20 strains

Anti-biotic	amount per disc	T20	T25	Mutant\ T20 A	Mutant T20 B
Amp.	10 μ g	R	R	R	R
Bac.	10 I.U.	R	R	R	R
Chl.	30 μ g	R	R	S	S
Ery.	15 μ g	R	R	R	R
Met.	5 μ g	R	R	R	R
Kan.	30 μ g	S	S	S	S
Pen.	5 I.U.	R	R	R	R
Rif.	5 μ g	S	S	S	S
Tet.	30 mcg	S	S	S	S

R = resistance
S = sensitivity

III were different by + 6 Kbp. It is possible that smaller fragments eluted off the gel. From the information on the gel, an average size of the plasmid was calculated and was estimated to be 44.05 Kbp. *Xba* I and *Sac* I generated fragments whose sizes totalled 39.1 and 38.65 Kbp, 10.04 and 12.02 Kbp smaller than the average. In both cases, the plasmid seems not to have been completely digested as the high molecular weight fragments remain at the top of the gel. In the case of *Hind* III, the digestion does not appear to have been incomplete since there were no high molecular weight fragments, however, the third band from the top of the gel, appears to have a greater intensity than the others. This suggests the presence of more than one fragment of identical size. This would then account for the smaller size.

3.3.3.3 Construction of the restriction digest map of *P. fluorescens* T20 plasmid DNA

Using a series of restriction endonucleases in pairs, together with the information derived from the single enzyme digests, it was possible to suggest a restriction endonuclease digest map of the plasmid obtained from *P. fluorescens* T20. After the digests were complete the fragments were electrophoresed on a 0.8% gel as

3.3.5 Analysis of the *P. fluorescens* T20 plasmid

3.3.5.1 Estimation of the molecular weight of the plasmid

It appeared that the plasmid occurring in T20 had a high molecular weight (refer to section 3.3.3). Subsequently, to estimate the molecular weight of the plasmid occurring in the T20 strain, it was necessary to carry out a series of single restriction endonuclease digests on the plasmid. The fragments which were derived from those digests were electrophoresed on a 0.8% agarose gel as is shown in Figure 3.6. Using *Hind* III digested bacteriophage lambda DNA as the molecular weight marker, it was possible to estimate the sizes of the fragments. The fragments generated from each restriction endonuclease digestion of the pT20 were totalled and are listed in Table 3.4. However *Sal* I, *Pst* I, *Eco*RI and *Bam* HI were different by + 6 Kbp. It is possible that smaller fragments eluted off the gel. From those fragments occurring on the gel, an average size of the plasmid was calculated and was estimated to be 44.06 Kbp. *Xba* I and *Sac* I generated fragments whose sizes totalled 39.1 and 32.65 Kbp, 10.04 and 12.02 Kbp smaller than the average. In both cases, the plasmid seems not to have been completely digested as the high molecular weight fragments remain at the top of the gel. In the case of *Hind* III, the digestion does not appear to have been incomplete since there were no high molecular weight fragments, however, the third band from the top of the gel, appears to have a greater intensity than the others. This suggests the presence of more than one fragment of identical size. This would then account for the smaller size.

3.3.5.2 Construction of the restriction digest map of *P. fluorescens* T20 plasmid DNA

Using a series of restriction endonucleases in pairs, together with the information derived from the single enzyme digests, it was possible to suggest a restriction endonuclease digest map of the plasmid obtained from *P. fluorescens* T20. After the digests were complete the fragments were electrophoresed on a 0.8% gel as

described in section 2.15. Figure 3.6 illustrates the fragments generated from single restriction endonuclease digests. Seven endonucleases were used and are listed in the accompanying figure legend. From the single digests it was possible to determine the number of restriction endonuclease sites that occurred on the plasmid for each of the enzymes as well as the sizes of each fragment generated.

Next double digest mapping was carried out. The fragments generated are shown in Figures 3.7 and 3.8. Generally, when pairs of restriction endonucleases are used the sites of one endonuclease are oriented with respect to restriction sites of a 'reference enzyme'. The best reference enzyme is one that generates only 2 or 3 sites on a circular DNA molecule. Once fixed reference sites are established, digestion of the DNA with pairs of restriction endonucleases can be performed and based on the sizes of the fragments generated, the digest sites of other restriction endonucleases can be determined with respect to the reference enzyme. Subsequently, from an analysis of all the data a physical map can be proposed. Unfortunately, with the exception of *Xba* I, no restriction enzyme gave fewer than five sites (see Figure 3.4). As a result, it was necessary to run all possible combinations of restriction endonuclease pairs used for the single digests. Fragments generated from the double digests are shown in Figures 3.7 and 3.8 and fragment sizes are listed in Tables 3.5 and 3.6. Because of the number of fragments generated, it was necessary to use a computer package, MAPC (refer to section 2.10), to generate the physical map of the plasmid, T20 which is illustrated in Figure 3.9.

3.3.6 Subcloning of the *P. fluorescens* T20 protease gene

3.3.6.1 Shotgun cloning of pT20 into pUC 12

The mutant strain, T20B, strongly indicated that the protease gene was plasmid encoded. Based on this, an attempt was made to subclone the protease gene into a cloning vector. As shown in Figure 3.6, the plasmid extracted from T20, which

Figure 3.6 Restriction endonuclease generated fragments of pT20

The plasmid isolated from T20 was purified on a CsCl gradient and restriction endonuclease digests were carried out as described in sections 2.11.2 and 2.14 respectively. The digested fragments were loaded on to a 0.8% agarose gel using Bromophenol blue as the tracking dye. Electrophoresis was carried out for 3.5 hours at 40 volts. **Hind III** digested lambda DNA was used as the molecular weight marker.

Lane 1 = **Sal I**
Lane 2 = **Pst I**
Lane 3 = **Bam HI**
Lane 4 = **Xba I**
Lane 5 = **Hind III**
Lane 6 = **Sac I**
Lane 7 = **Eco RI**
Lane 8 = **Hind III** digested lambda
DNA

Figure 3-6: Restriction endonuclease generated fragments of pT20

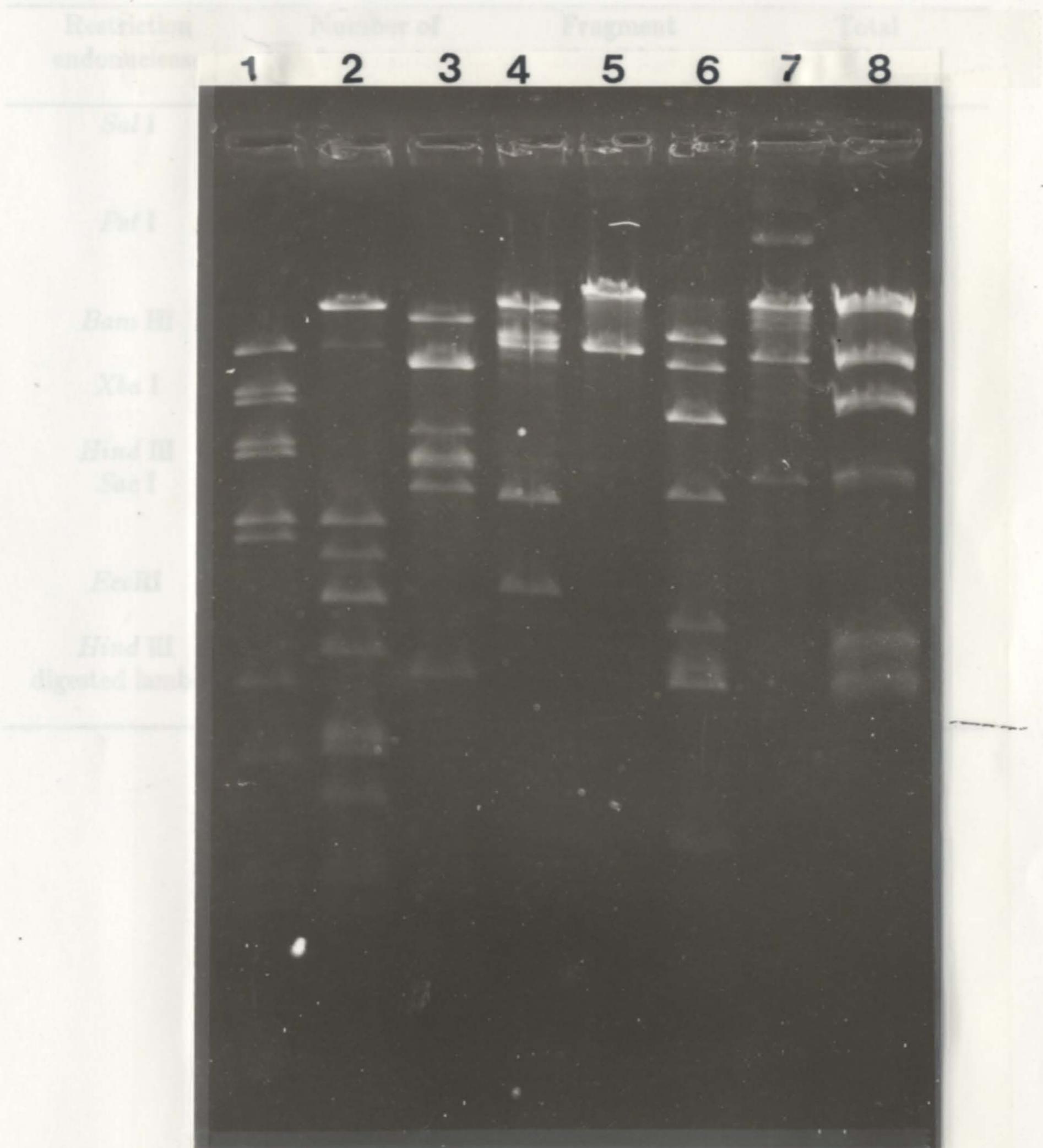


Table 3-4: Molecular weight of fragments derived from single restriction endonuclease digests of the T20 plasmid

Restriction endonuclease	Number of fragments	Fragment sizes(kbp)	Total Kbp
<i>Sal I</i>	9	10.65, 8.5, 7.4, 5.0, 4.4, 3.3, 2.8, 1.85, 1.65	45.55
<i>Pst I</i>	8	19.0, 10.9, 3.1, 2.6, 2.2, 1.95, 1.70, 1.60	43.05
<i>Bam HI</i>	6	13.5, 10.25, 5.50, 4.40, 3.70, 1.85	39.2
<i>Xba I</i>	5	17.80, 11.30, 10.70, 2.90, 2.25	44.95
<i>Hind III</i>	2	22.0, 10.65	32.65
<i>Sac I</i>	7	10.95, 10.10, 5.80, 3.40, 2.00, 1.85, 1.80	34.1
<i>EcoRI</i>	5	17.0, 12.5, 11.7, 10.20, 3.70	55.1
<i>Hind III</i> digested lambda	6	23.6, 9.636, 6.636, 4.333, 2.57, 1.985	48.76

Figure 3.7 Restriction endonuclease generated fragments from double digestions of the plasmid isolated from *P. fluorescens* T20

Plasmid DNA was isolated and purified from T20 as described in the procedure outlined in section 2.11.2. Digestion of the plasmid DNA was carried out as described in section 2.14, except that volume of the restriction digests remained the same except the amount of water used was adjusted accordingly. The fragments were loaded on to a 0.8% agarose gel and electrophoresis was carried out for 3 hours at 40 volts.

Lane 1 = Pst I/ Bam HI
Lane 2 = Pst I/ Xba I
Lane 3 = Pst I/ Hind III
Lane 4 = Pst I/ Sal I
Lane 5 = Pst I/ Eco RI
Lane 6 = Hind III/ Sal I
Lane 7 = Hind III/ Eco RI
Lane 8 = Hind III digested lambda
DNA

Figure 3-7: Restriction endonuclease generated fragments from double digestions of the plasmid isolated from *P. fluorescens* T20

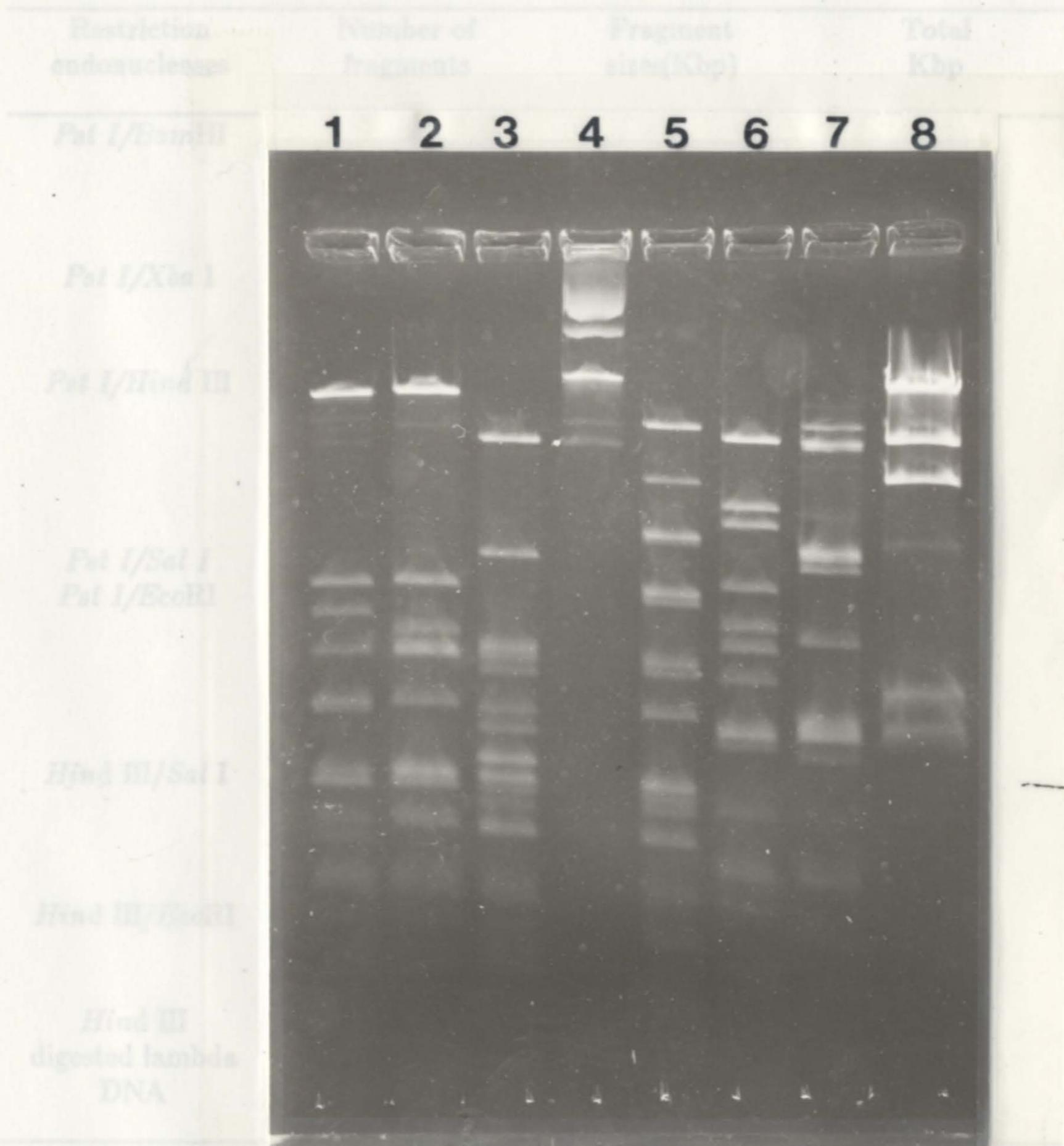


Table 3-5: The molecular weights of fragments derived from double restriction enzyme digests of the T20 plasmid

Restriction endonucleases	Number of fragments	Fragment sizes(Kbp)	Total Kbp
<i>Pst I/BamHI</i>	10	29.0, 3.20, 2.80, 2.40, 2.0, 1.75, 1.70, 1.60, 1.55, 1.35	47.35
<i>Pst I/Xba I</i>	9	29.0, 3.20, 2.60, 2.35, 2.00, 1.75, 1.70, 1.55, 1.35	45.45
<i>Pst I/Hind III</i>	13	10.15, 4.10, 2.45, 2.35, 2.25, 2.00, 1.95, 1.75, 1.70, 1.65, 1.60, 1.50, 1.25	34.7
<i>Pst I/Sal I</i>	3	33.00, 12.00, 10.15	55.15
<i>Pst I/EcoRI</i>	13	10.80, 6.80, 4.30, 3.00, 2.80, 2.45, 2.20, 1.95, 1.65, 1.60, 1.55, 1.45, 1.25	41.8
<i>Hind III/Sal I</i>	11	10.15, 5.20, 4.50, 3.10, 2.50, 2.35, 2.15, 1.85, 1.80, 1.50, 1.35	36.45
<i>Hind III/EcoRI</i>	9	10.80, 10.00, 7.80, 6.60, 3.60, 3.35, 2.40, 1.75, 1.25	47.55
<i>Hind III</i> digested lambda DNA	6	23.6, 9.636, 6.636, 4.333, 2.257, 1.985	48.447

Figure 3.8 Restriction endonuclease generated fragments from the double digestion of the T20 plasmid

The plasmid DNA was isolated and purified as outlined in section 2.11.1. As previously described, digestion of the plasmid DNA was carried out using pairs of restriction endonucleases. As before, the fragments were loaded on to a 0.8% agarose gel and electrophoresis was carried out for 3 hours at 40 volts as described in section 2.16.

Lane 1 = Sa1 1/Eco R1
Lane 2 = Sa1 1/Bam H1
Lane 3 = Sa1 1/ Xba 1
Lane 4 = Sa1 1/Sac 1
Lane 5 = Bam H1/Xba 1
Lane 6 = Bam H1/Hind 111
Lane 7 = Bam H1/Sac 1
Lane 8 = Hind 111 digested
lambda DNA

Table 3-8: Restriction endonuclease generated
Figure 3-8: Restriction endonuclease generated fragments from the double digestion of the T20 plasmid

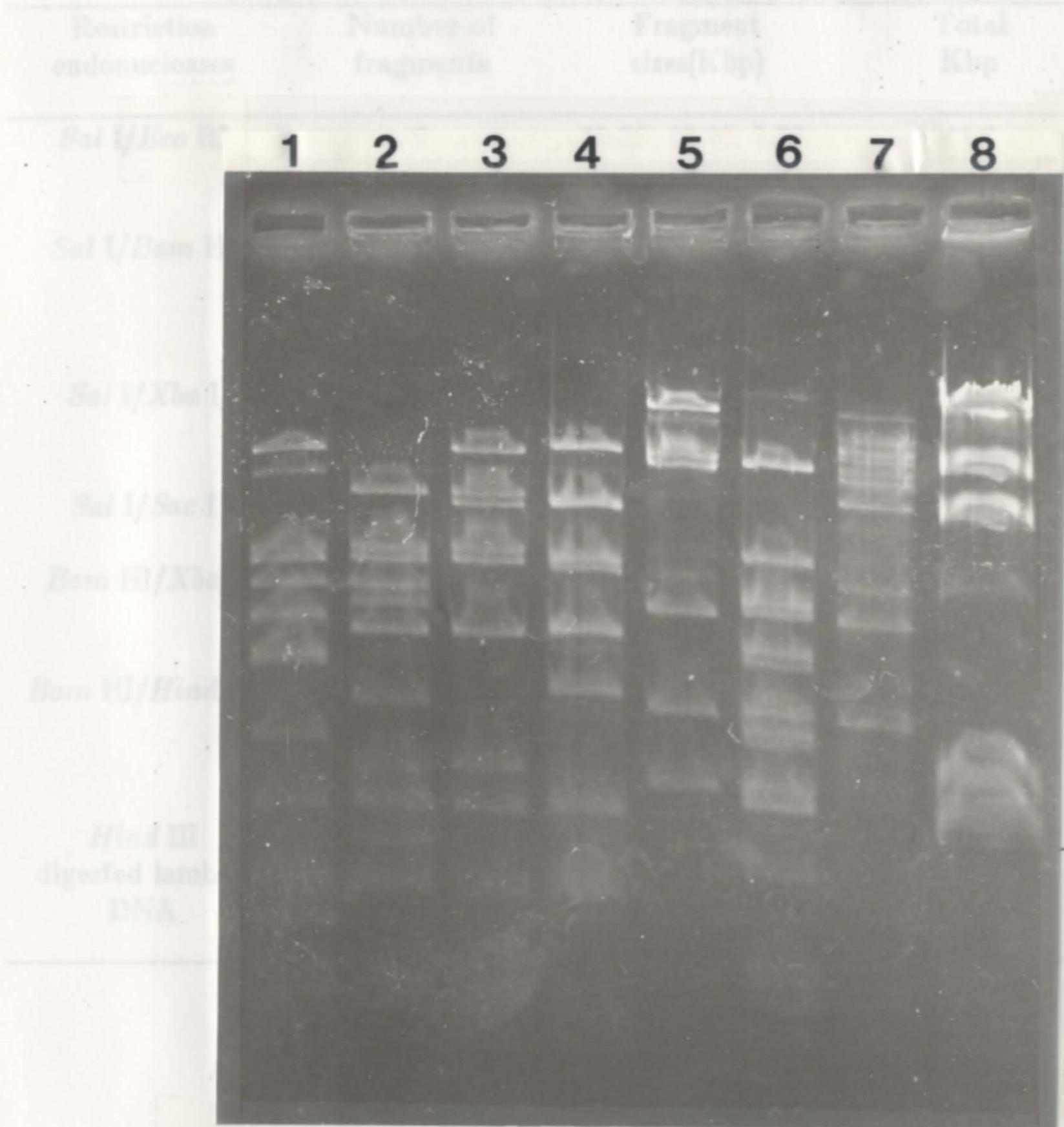


Table 3-6: Restriction endonuclease generated fragments from double digests of pT20

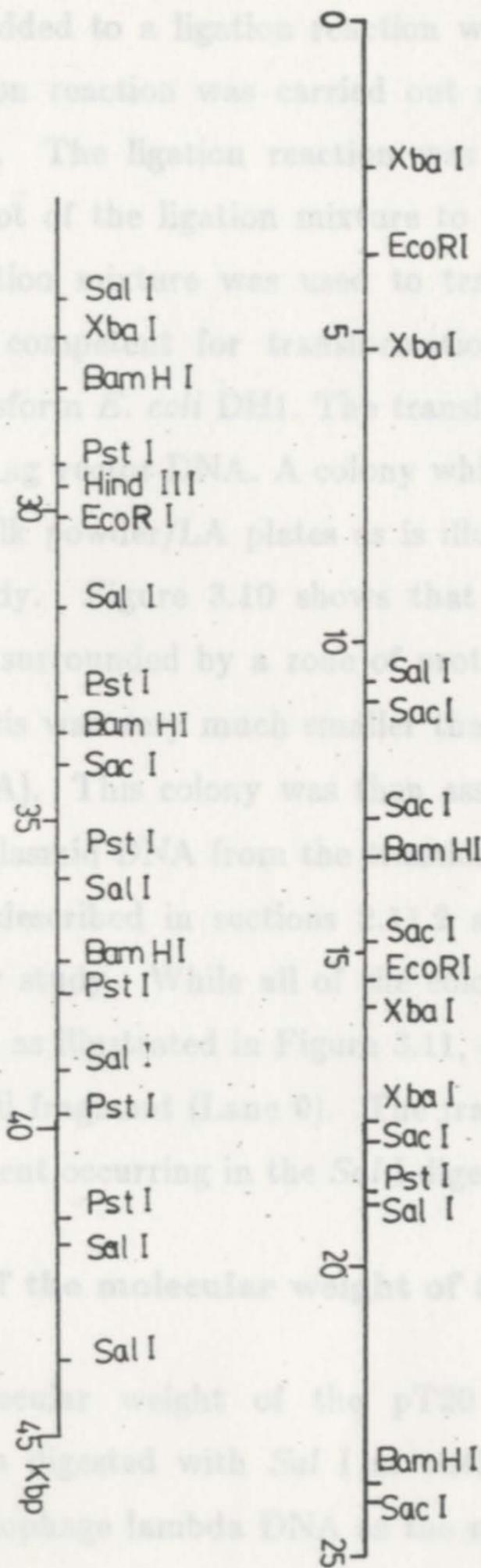
Restriction endonucleases	Number of fragments	Fragment sizes(Kbp)	Total Kbp
<i>Sal I/Eco RI</i>	9	11.35, 10.20, 5.30, 4.60, 3.90, 3.10, 2.30, 1.95, 1.50	44.2
<i>Sal I/Bam HI</i>	11	9.20, 5.80, 5.20, 4.60, 4.20, 3.60, 2.70, 2.10, 1.95 1.80, 1.60	42.75
<i>Sal I/Xba I</i>	8	11.00, 9.20, 8.00, 5.50, 5.20, 3.50, 2.15, 1.95	46.5
<i>Sal I/Sac I</i>	6	11.00, 8.00, 5.20, 3.50, 2.70, 2.10	32.5
<i>Bam HI/Xba I</i>	8	10.60, 10.35, 9.60, 5.10, 4.80, 3.90, 2.55, 2.00	48.9
<i>Bam HI/Hind III</i>	11	10.20, 5.00, 3.80, 3.50, 3.15, 3.00, 2.45, 2.30, 1.95, 1.90, 1.60	38.85
<i>Hind III</i> digested lambda DNA	6	23.606, 9.636, 6.636 4.333, 2.567, 1.985	48.763

Figure 3-9: Restriction digest map of the T20 plasmid

was cut with *Sal*I, gave rise to two fragments. They ranged in size from 1.7 to 6.6 Kbp. Since there were a number of small fragments generated, i.e. less than 5Kbp, there was a better chance of cloning one of the fragments into a vector. These fragments were added to a ligation reaction with dephosphorylated *Sal*I cut pUC12. The ligation reaction was carried out according to the procedure outlined in section 2.13. The ligation reaction shows to be complete by subjecting a small aliquot of the ligation mixture to agarose gel electrophoresis. Following this, the ligation mixture was used to transform *E. coli* DH1. To ensure that cells were competent for transformation, pKT300, a broad host vector, was used to transform *E. coli* DH1. The transformation efficiency was 3.2×10^8 transformants per μ g DNA. A colony which gave a positive protease reaction on 2% skim milk LA plates is illustrated in Figure 3.10 was selected for further study. Figure 3.10 shows that the growth from a single subcultured colony, was surrounded by a zone of proteolysis as observed in plate B. The zone of proteolysis was much smaller than that occurring around *P. fluorescens* T20 (Plate A). This colony was assayed for the presence of a *Sal*I pT20 fragment. Plasmid DNA from the mixed colonies was prepared and electrophoresed as described in sections 2.12 and 2.13. Twelve colonies were selected for further study. While all colonies were surrounded by a small zone of proteolysis, as illustrated in Figure 3.11, only one colony showed the presence of a single small fragment (Lane 9). This fragment corresponded to the 1.4 or the 3.3 Kbp fragment occurring in the *Sal*I digested pT20 (Lane 1).

3.3.6.3 Estimation of the molecular weight of the pT20 insert

To estimate the molecular weight of the pT20 insert, the pT20/pUC12 recombinant, pUT3, was digested with *Sal*I as outlined in section 2.13. Using *Hind*III digested bacteriophage lambda DNA as the molecular weight marker, it was possible to estimate the molecular weight of the fragment (refer to Figure 3.12) which corresponded to the 3.3 Kbp fragment in *Sal*I digested pT20 (refer to Figure 3.6 and Figure 3.11).



was cut with *Sal* I, gave rise to nine fragments. They ranged in size from 1.7 to 9.6 Kbp. Since there were a number of small fragments generated, i.e. less than 5Kbp, there was a better chance of cloning one of the fragments into a vector. These fragments were added to a ligation reaction with dephosphorylated *Sal* I cut *pUC12*. The ligation reaction was carried out according to the procedure outlined in section 2.13. The ligation reaction was shown to be complete by subjecting a small aliquot of the ligation mixture to agarose gel electrophoresis. Following this, the ligation mixture was used to transform *E. coli* DH1. To ensure that cells were competent for transformation, *pKT230*, a broad host vector, was used to transform *E. coli* DH1. The transformation efficiency was 3.2×10^6 transformants per μg vector DNA. A colony which gave a positive protease reaction on 2% skim milk powder/LA plates as is illustrated in Figure 3.10 was selected for further study. Figure 3.10 shows that the growth from a single subcultured colony, was surrounded by a zone of proteolysis as observed in plate B. The zone of proteolysis was very much smaller than that occurring around *P. fluorescens* T20 (Plate A). This colony was then assayed for the presence of a *Sal* I *pT20* fragment. Plasmid DNA from the transformed colonies was prepared and electrophoresed as described in sections 2.11.2 and 2.13. Twelve colonies were selected for further study. While all of the colonies were surrounded by a small zone of proteolysis, as illustrated in Figure 3.11, only one colony showed the presence of a single small fragment (Lane 9). The fragment corresponded to the 4.6 or the 3.3 Kbp fragment occurring in the *Sal* I digested *pT20* (Lane 1).

3.3.6.2 Estimation of the molecular weight of the *pT20* insert

To estimate the molecular weight of the *pT20* insert, the *pT20/pUC12* recombinant, *pUT8*, was digested with *Sal* I as outlined in section 2.13 Using *Hind* III digested bacteriophage lambda DNA as the molecular weight marker, it was possible to estimate the molecular weight of the fragment (refer to Figure 3.12) which corresponded to the 3.3 Kbp fragment in *Sal* I digested *pT20* (refer to Figure 3.6 and Figure 3.11).

Figure 3.10 Proteolytic reaction of pT20/pUC 12 transformed E. coli DHI on casein plates

The plasmid DNA isolated from T20 was cut with **Sal I** and ligated to **Sal I** cut pUC 12 as described in section 2.14. The ligated DNA was subsequently transformed into **E. coli** DHI as described in section 2.13. The transformed cells were then plated out on to Luria agar supplemented with 2% casein and 25 ug/mL of ampicillin.

Plate A = **P. fluorescens** T20

Plate B = **E. coli** DHI transformed with
pUT 8

Plate C = T20 A mutant strain

Figure 3-10: Proteolytic reaction of pT20/pUC 12 transformed *E. coli* DH1 on casein plates

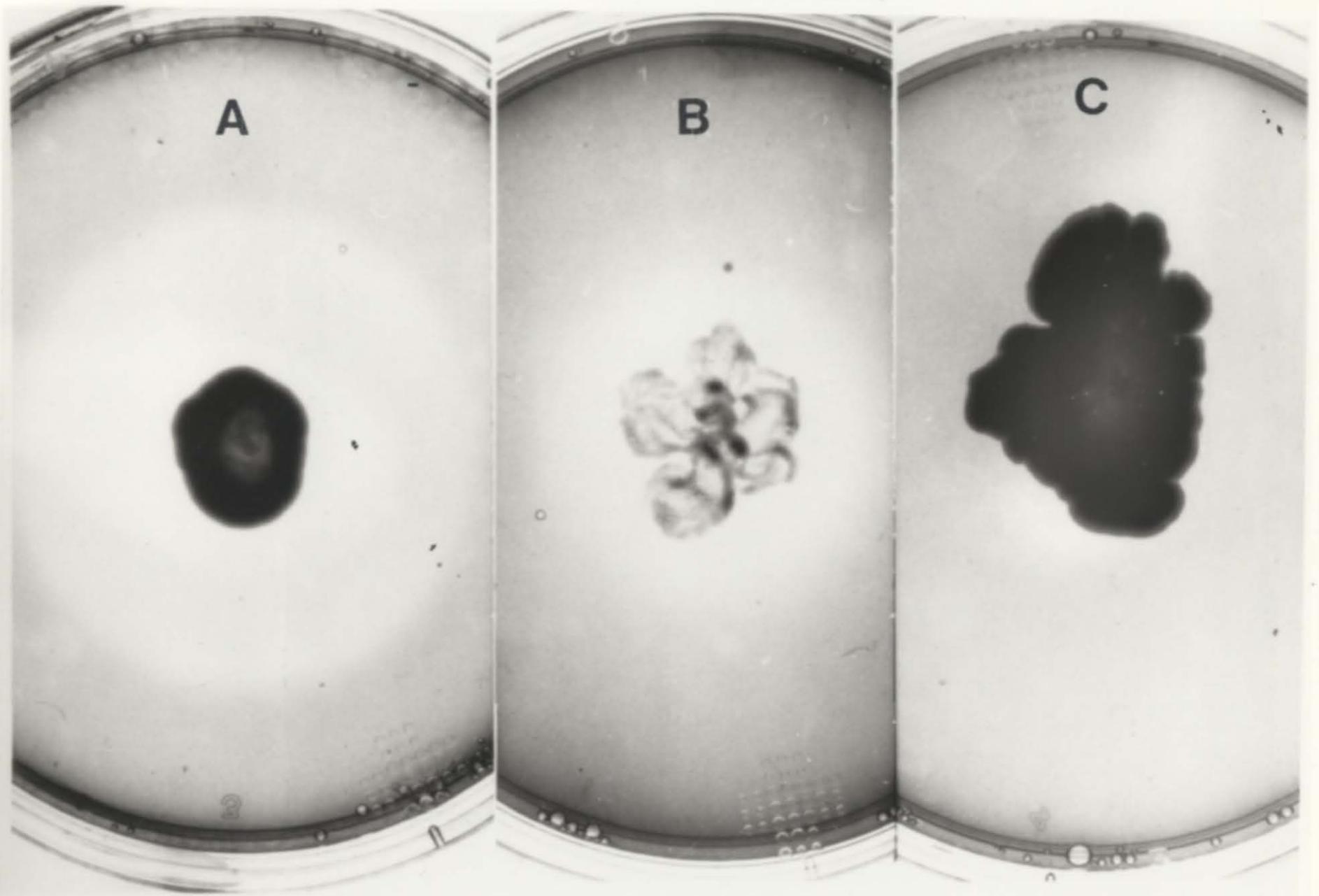
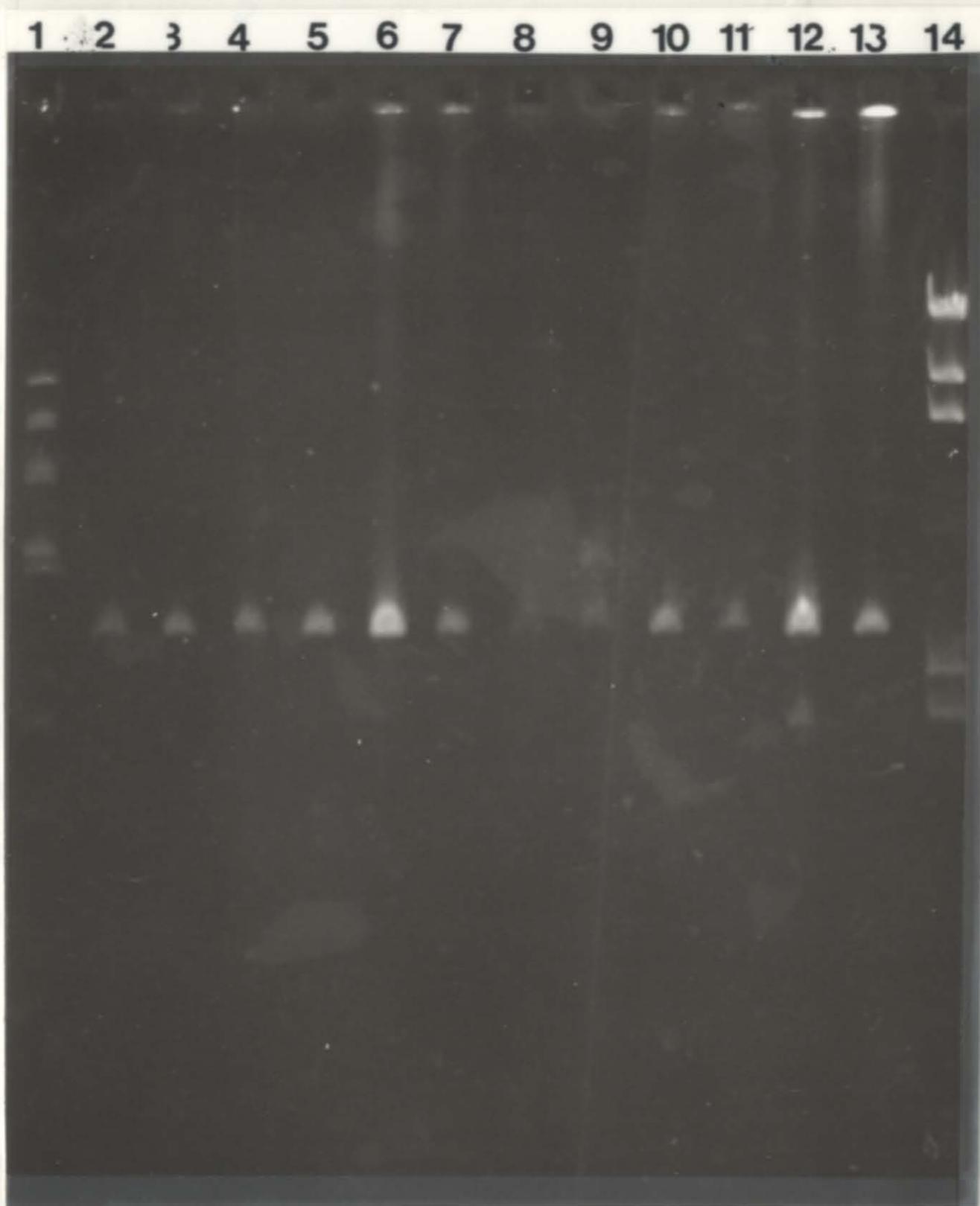


Figure 3.11 Screening of twelve different colonies for Sal I pT20 inserts cloned into the vector pUC 12

Using the small scale or mini-prep method outlined in section 2.11.1, plasmid was isolated from 12 colonies which did show small zones of proteolysis. The plasmids were loaded on to 0.8% agarose gel and electrophoresed at 40 volts for 3 hours.

Lane 1 = Sal I digested pT20
Lane 2-8 = Sal I digested pUC 12
Lane 9 = Sal I digested pUT 8
Lane 10-13 = Sal I digested pUC 12
Lane 14 = Hind III digested lambda

Figure 3-11: Screening of twelve different colonies for *Sal* I pT20 inserts cloned into the vector pUC 12



contains *Sal* I cut pUC12 DNA did not hybridize to the probe, thus indicating that the cloning vector, pUC 12, did not share homologous regions with the plasmid derived from T20 (pT20). In each of wells 2 to 6, fragments derived from single and double digests of the pT20 insert cloned into the vector pUC 12, did hybridize with the probe, ^{32}P labelled pT20. Thus, the probe hybridized only with the 3.3 Kbp fragment derived from the *Sal* I cut T20 plasmid and shared no homologous regions with the vector, pUC 12.

3.3.6.3 Construction of a physical map of the *Sal* I insert cloned into the vector pUC12 The physical map of *Sal* I was constructed using a series of single and double restriction endonuclease digests. As before, the molecular weights of the fragments generated were estimated using a calibration curve of the log molecular weights of *Hind* III digested bacteriophage lambda DNA versus their migration distance. Figure 3.12 shows the fragments resulting from single enzyme restriction digests. The sizes of the fragments generated are listed in Table 3.7. *Sal* I was used as the reference enzyme and double digests were run using pairs of restriction endonucleases as described in section 2.14. The fragments generated from the double digests are shown in Figure 3.13 and the fragment sizes are listed in Table 3.8. Using the MAPL package and the sizes of the fragments generated from both the single and double restriction digests, it was possible to determine a physical map of the pT20 insert region as is illustrated in Figure 3.14. Those fragments which clearly represented undigested products and are indicated in the tables were excluded.

3.3.6.4 Southern blotting To ensure that the fragment cloned actually belonged to pT20, it was necessary to carry out a Southern blot analysis. The pT20 plasmid was labelled with ^{32}P and was used to probe pUT8 DNA. Figure 3.15 illustrates the fragments resulting from the single enzyme digestion of pUC12 DNA and single and double enzyme digests of pUT8 as they appear following separation by electrophoresis on a 0.8% agarose gel. This gel was used as the source of material to be blotted and probed with the labelled pT20 plasmid. Figure 3.16 shows the result of the blotting and hybridization. Well 1, which contains *Sal* I cut pUC12 DNA did not hybridize to the probe, thus indicating that the cloning vector, pUC 12, did not share homologous regions with the plasmid derived from T20 (pT20). In each of wells 2 to 6, fragments derived from single and double digests of the pT20 insert cloned into the vector pUC 12, did hybridize with the probe, ^{32}P labelled pT20. Thus, the probe hybridized only with the 3.3 Kbp fragment derived from the *Sal* I cut T20 plasmid and shared no homologous regions with the vector, pUC 12.

Figure 3-12: Fragments derived from single restriction endonuclease digestions of pOT 8

Plasmid DNA was isolated and purified from pOT8 as described in the procedure outlined in section 2.1.2. Digestion of the plasmid DNA was carried out as described in section 2.4. The fragments were loaded on to a 3.8% agarose gel and electrophoresis was carried out for 3 hours at 40 volts.

Lane 1 - Hind III digested lambda
DNA
Lane 2 = Pst I
Lane 3 = Bam HI
Lane 4 = Xba I
Lane 5 = Hind III
Lane 6 = Sal I
Lane 7 = Sac II
Lane 8 = undigested pOT 8

Figure 3.12: Fragments derived from single restriction endonuclease digestions of pUT 8

Plasmid DNA was isolated and purified from pUT8 as described in the procedure outlined in section 2.11.2. Digestion of the plasmid DNA was carried out as described in section 2.4. The fragments were loaded on to a 0.8% agarose gel and electrophoresis was carried out for 3 hours at 40 volts.

- Lane 1 = **Hind III** digested lambda DNA
- Lane 2 = **Pst I**
- Lane 3 = **Bam HI**
- Lane 4 = **Xba I**
- Lane 5 = **Hind III**
- Lane 6 = **Sal I**
- Lane 7 = **Eco RI**
- Lane 8 = undigested pUT 8

Figure 3-12: Fragments derived from single restriction endonuclease digestions of pUT 8

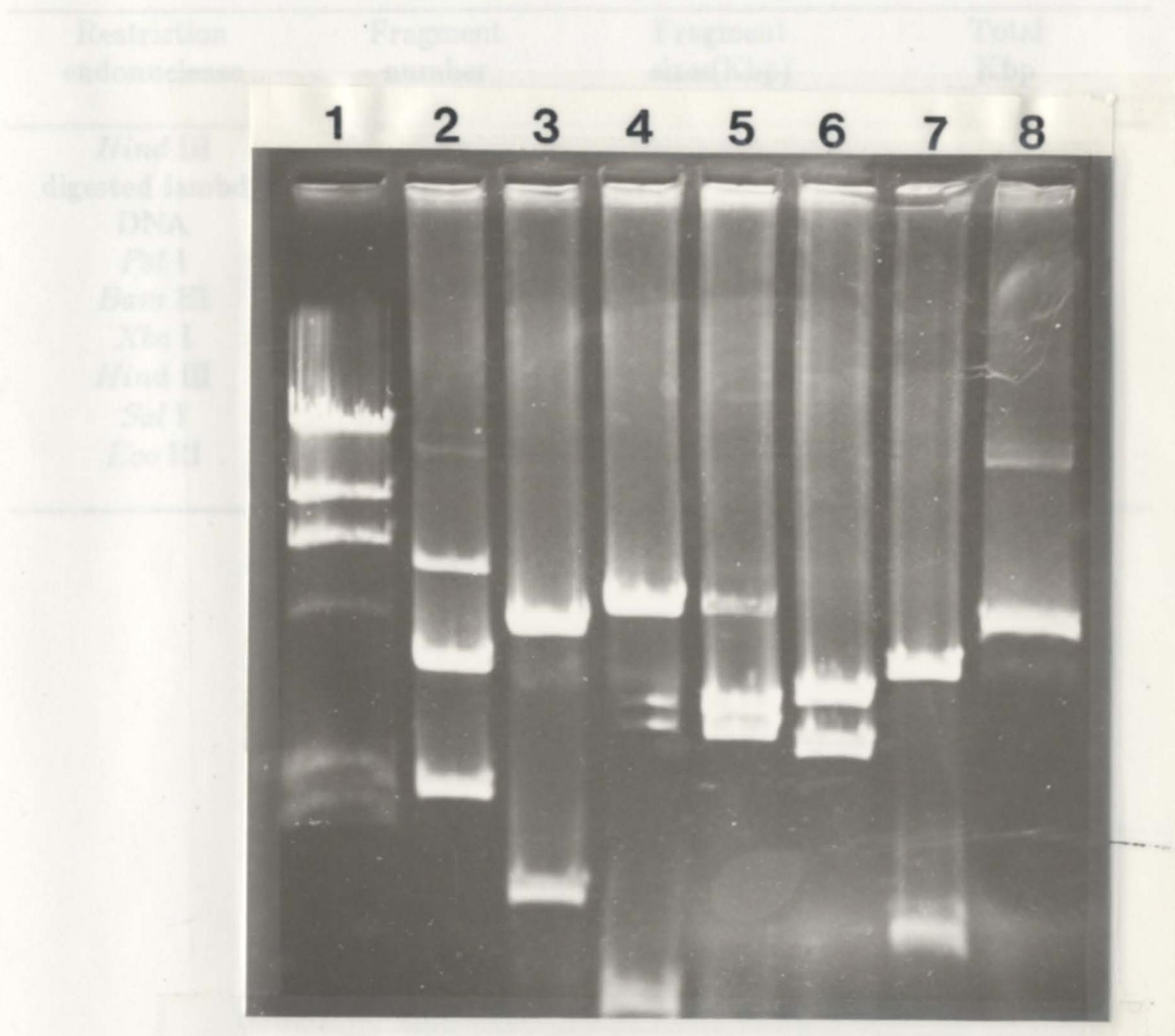


Table 3-7: Fragment sizes of the single restriction endonuclease digestion of pUT 8

Restriction endonuclease	Fragment number	Fragment sizes(Kbp)	Total Kbp
<i>Hind</i> III digested lambda DNA	6	23.606, 9.636, 6.636 4.333, 2.562, 1.985	
<i>Pst</i> I	3	5.80, 3.30, 1.90	11.0
<i>Bam</i> HI	2	4.00, 1.50	5.5
<i>Xba</i> I	2	4.50, 1.25	5.75
<i>Hind</i> III	2	4.50, 2.70, 2.40	9.6
<i>Sal</i> I	2	3.3, 2.40	5.2
<i>Eco</i> RI	2	3.10, 1.40	4.5

**Figure 3.13 Double restriction endonuclease digests of
of the recombinant plasmid pUT 8**

The plasmid DNA pUT 8 was isolated and digested as described in sections 2.12 and 2.14 respectively. The digested pDNA fragments were electrophoresed on a 0.8% agarose gel as described in section 2.16.

Lane 1 = Sal I/ Pst I
Lane 2 = Sal I/ Eco RI
Lane 3 = Sal I/ Hind III
Lane 4 = Sal I/ BamHI
Lane 5 = Sal I/ Xba I
Lane 6 = Hind III digested lambda
DNA

Figure 3-13: Double restriction endonuclease digests of the recombinant plasmid pUT 8

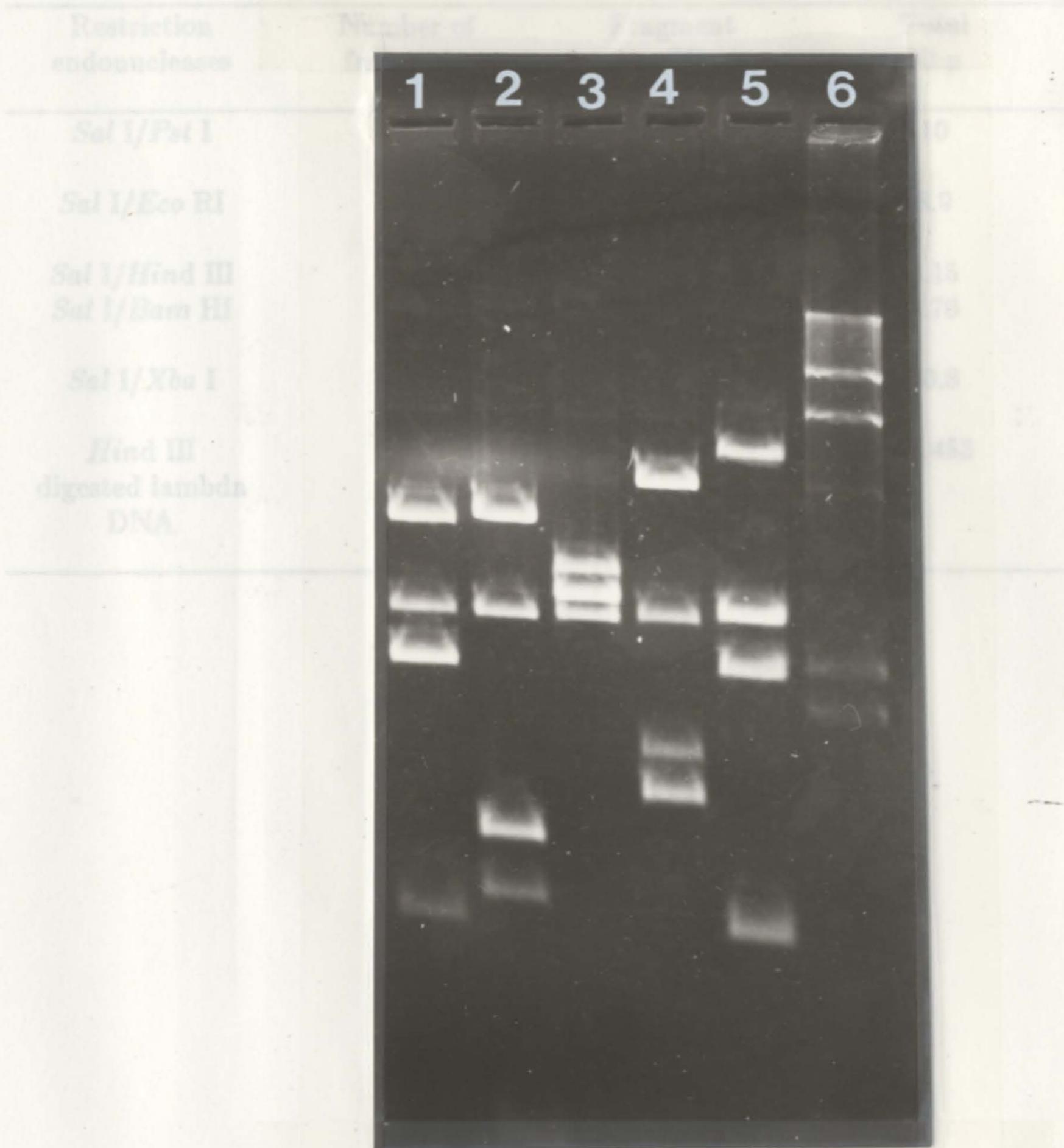


Table 3-8: Fragments generated from double restriction endonuclease digestions of pUT 8

Restriction endonucleases	Number of fragments	Fragment sizes(Kbp)	Total Kbp
<i>Sal I/Pst I</i>	4	3.9, 2.7, 2.2, 1.2	10
<i>Sal I/Eco RI</i>	4	3.9, 2.5, 1.3, 1.2	8.9
<i>Sal I/Hind III</i>	3	3.0, 2.7, 2.45,	8.15
<i>Sal I/Bam HI</i>	4	4.26, 2.50, 1.6, 1.4	9.76
<i>Sal I/Xba I</i>	4	5.1, 2.5, 2.0, 1.2	10.8
<i>Hind III</i> digested lambda DNA	6	23.606, 9.636, 6.636 4.333, 2.257, 1.985	48.453

Figure 3.14 Physical map of the Sal I pT20 insert in pUC12

Using the fragment sizes of the single and double restriction digests and the MAPC sequence program, it was possible to determine the orientation of the restriction endonuclease sites in the **Sal I** pT20 insert.

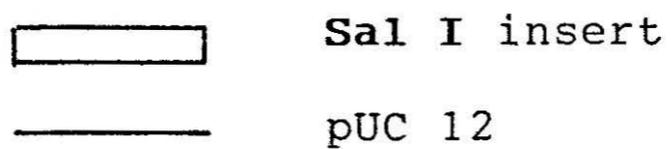
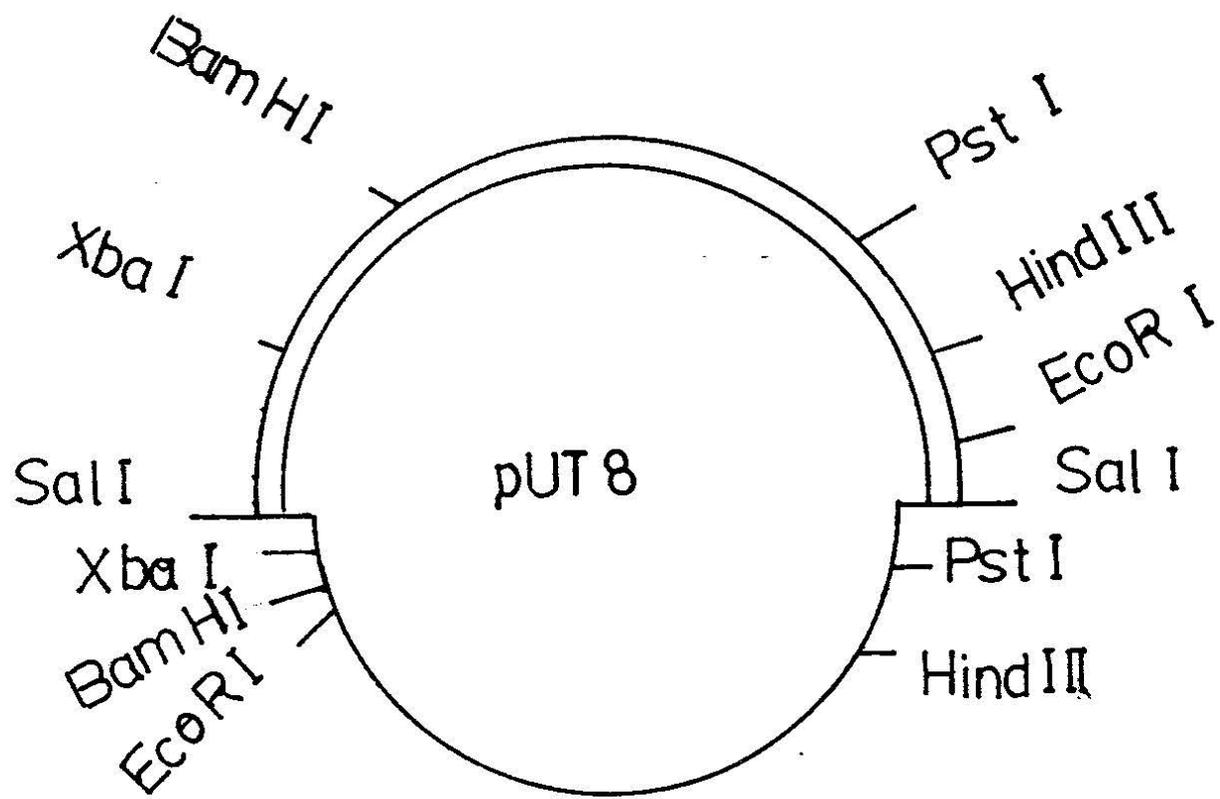


Figure 3-14: Physical map of the *Sal* I pT20 insert in pUC12



In order to determine whether the fragment cloned was present in more than one copy and where that fragment occurred in the physical map of pT20, it was necessary to probe pT20 with ^{32}P labelled pUT8. Representative single and double enzyme digests of pT20 were prepared and the fragments separated on a 0.8% agarose gel as illustrated in Figure 3.17. Those fragments which were complementary to the pUT 8 *Sal* I insert hybridized to the radiolabelled probe as shown in Figure 3.18. In fact, it can be seen from the Southern blot, that the *Sai* I gene sequence occurring in pUT 8 is not unique since the radiolabelled probe hybridized to more than one region of pT20. As was observed in Figure 3.18, the probe hybridized strongly with several fragments generated from *Sal* I digested pT20 (Lane 1), they include fragments which are 9.15, 8.8, 5.45, and 3.3 Kbp in length. In Lane 2, two fragments generated from *Pst* I digestion hybridized with the probe. They were 2.6 and 1.75 Kbp. Two fragments, 9.95, and 2.0 Kbp, generated from an *Eco* RI digestion. From the *Bam* HI digestion, fragments 9.3 and 8.9 Kbp hybridized with the probe. Hybridization was very efficient since the probe hybridized even to those fragments where only a small DNA sequence of the pT20 insert could have remained attached to the vector after digestion as shown by faint band occurring in Lanes 3, 4, 9 and 12 of Figure 3.18 (indicated by the arrow).

3.3.7 Immunoprecipitation of the intracellular and extracellular extracts of pUT 8

Intracellular and extracellular extracts of JM83 carrying the recombinant plasmid, pUT 8 were used to determine, by immunoprecipitation whether the protease observed on the milk powder plates was, in fact, the heat-stable T20 protease. The antibody used was that which was raised against the purified protease produced by *P. fluorescens* T25, which had been shown previously to cross react with the T20 protease (Jackman, *et al.*, 1983). As is illustrated in Figure 3.19 in well C and well D, pUT 8 showed no immunoprecipitation with the T25 antibody.

Figure 3.15 Fragments generated from single and double enzyme digests of pUT 8

As previously described, the fragments resulting from double enzyme digests of pUT 8 were prepared according to the procedure outlined in section 2.14. Electrophoresis of the fragments was carried out for 3.5 hours at 40 volts.

- Lane 1 = Sal I digested pUC 12
- Lane 2 = Sal I digested pUT 8
- Lane 3 = Sal I/ Pst I
- Lane 4 = Sal I/ Eco RI
- Lane 5 = Sal I/ Hind III
- Lane 6 = Sal I/ Bam HI
- Lane 7 = Sal I/ Xba I
- Lane 8 = Hind III digested
lambda DNA

Figure 3-15: Fragments generated from single and double enzyme digests of pUT 8

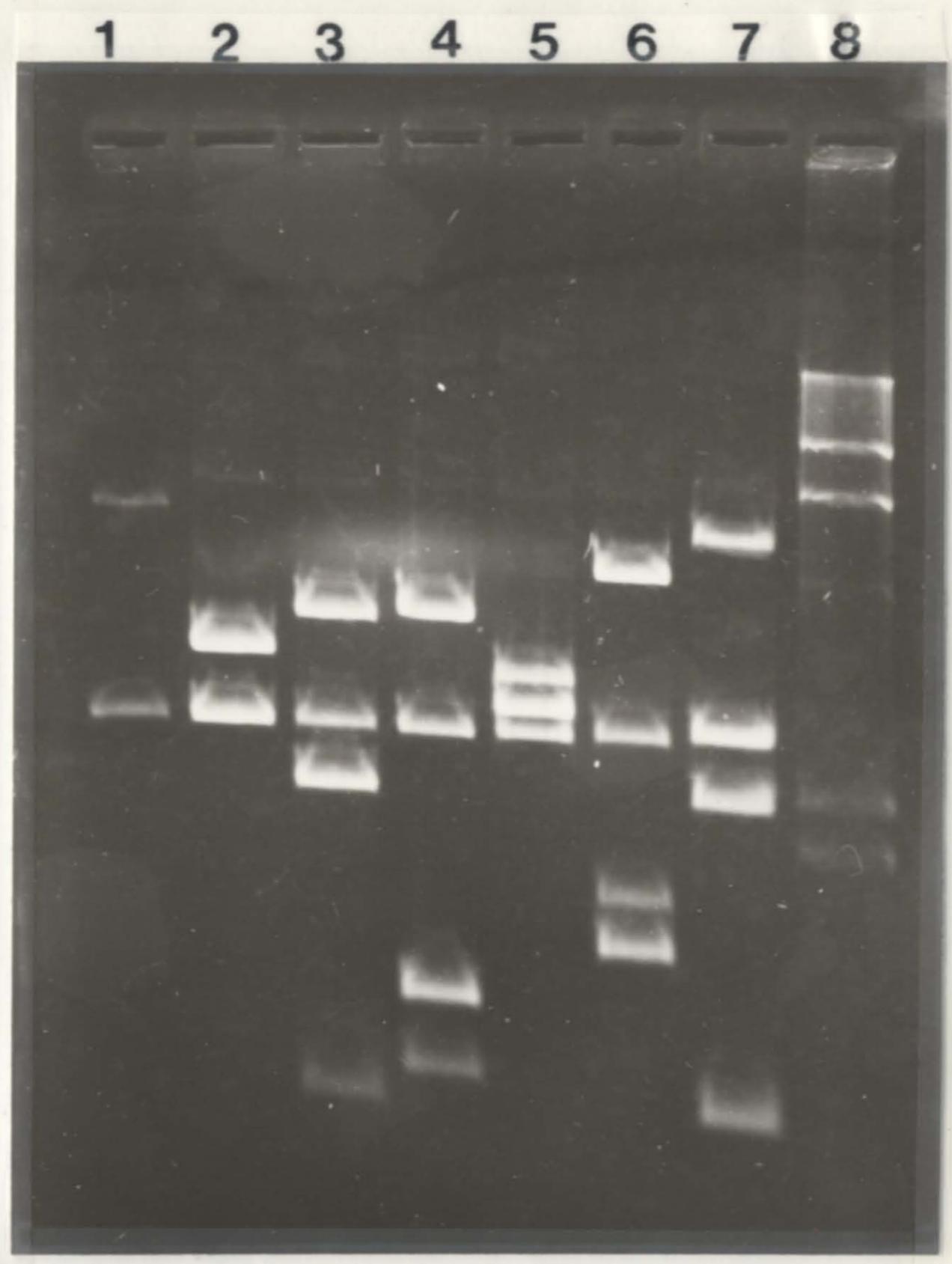


Figure 3.16 Southern Blot of fragments generated from single and double digests of pUT 8 with ³²P labelled pT20

DNA fragments electrophoretically separated on a 0.8% agarose gel, were immobilized on nitrocellulose filters according to the method described in section 2.18. The T20 plasmid, radioactively labelled with ³²P was used to probe the pUT 8 fragments bound to the nitrocellulose membrane.

Lane 1 = **Sal I** digested pUC 12
Lane 2 = **Sal I** digested pUT 8
Lane 3 = **Sal I/ Pst I**
Lane 4 = **Sal I/ Eco RI**
Lane 5 = **Sal I/ Hind III**
Lane 6 = **Sal I/ Bam HI**
Lane 7 = **Sal I/ Xba I**
Lane 8 = **Hind III** digested lambda
DNA

The arrow indicates the position of the 3.3 Kbp fragment in Lane 2.

Figure 3-16: Southern blot of fragments generated from single and double digests of pUT 8 with ^{32}P labelled pT20

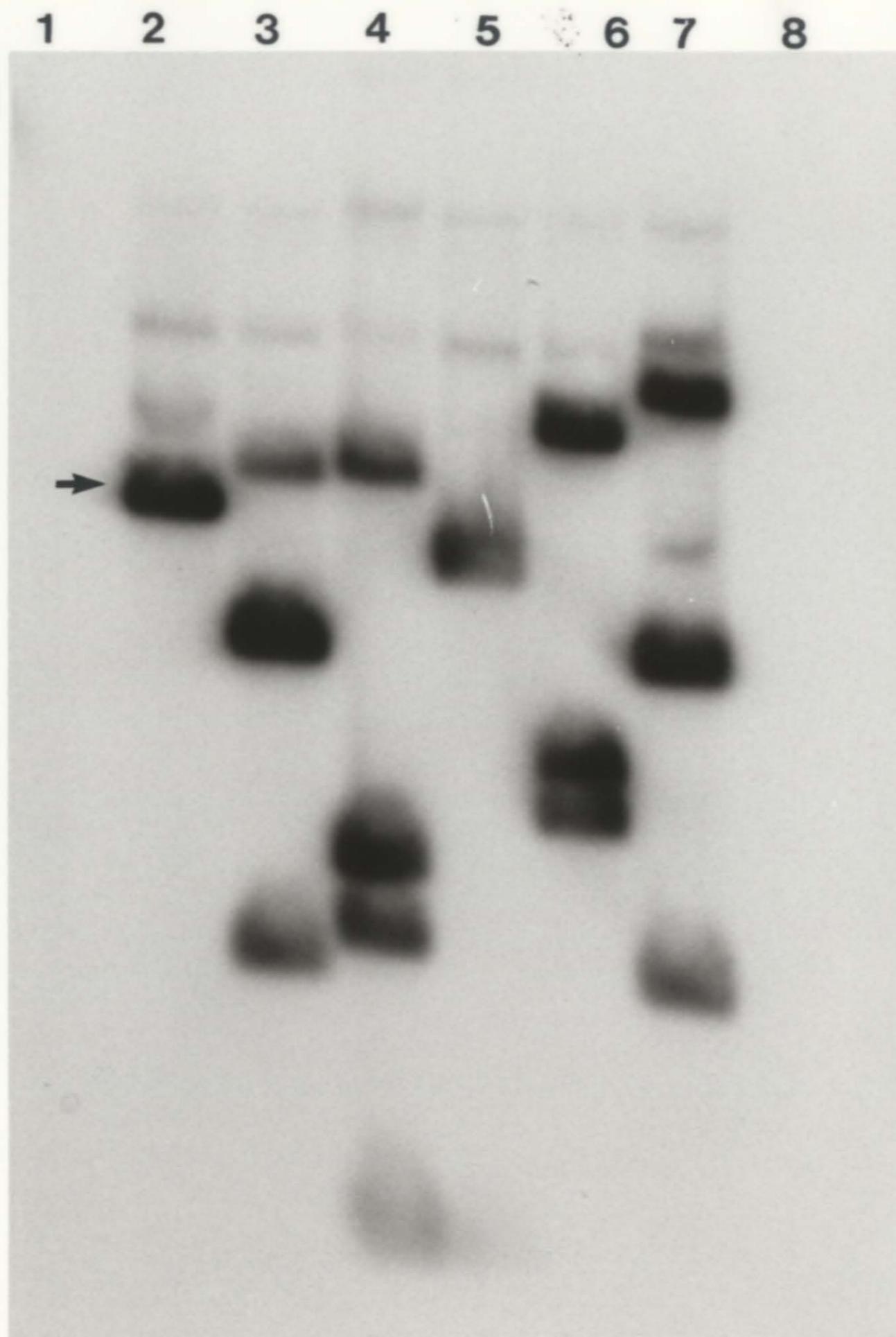


Figure 3.17 Fragments of pT20 generated by single and double restriction endonuclease digests

Plasmid DNA isolated from the T20 strain was purified on a cesium chloride gradient as outlined in section 2.12. The purified DNA was digested in both single and double restriction endonuclease reactions and the fragments generated from those reactions were run on a 0.8% agarose gel as described in sections 2.14 and 2.16 respectively.

- Lane 1 = Sal I
- Lane 2 = Pst I
- Lane 3 = Eco RI
- Lane 4 = Hind III
- Lane 5 = Bam HI
- Lane 6 = Xba I
- Lane 7 = Hind III digested lambda DNA
- Lane 8 = Sal I/ Pst I
- Lane 9 = Sal I/ Eco RI
- Lane 10 = Sal I/ Hind III
- Lane 11 = Sal I/ Bam HI
- Lane 12 = Sal I/ Xba I

Figure 3-17: Fragments of pT20 generated by single and double restriction endonuclease digests



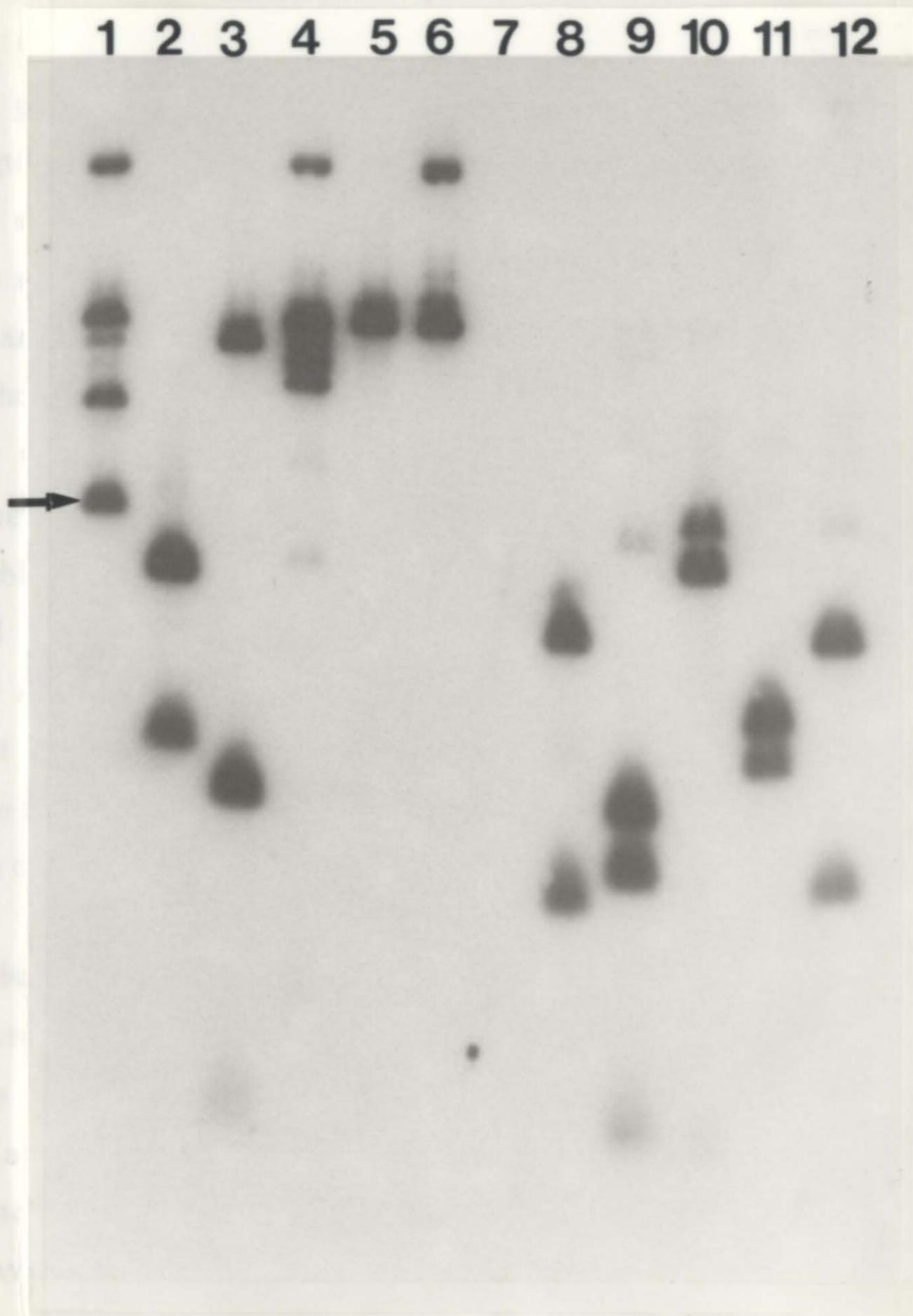
Figure 3.18 Southern blot of the T20 plasmid using ^{32}P labelled pUT 8 as the probe

The fragments generated from pT20 were electrophoretically separated on a 0.8% agarose gel as described in section 2.16. They were then immobilized on nitrocellulose filters as outlined in section 2.18. The plasmid referred to as pUT 8 was labelled with ^{32}P and used to probe the pT20 fragments bound to the nitrocellulose membrane.

Lane 1 = Sal I
Lane 2 = Pst I
Lane 3 = Eco RI
Lane 4 = Hind III
Lane 5 = Bam HI
Lane 6 = Xba I
Lane 7 = Hind III digested lambda
DNA
Lane 8 = Sal I/ Pst I
Lane 9 = Sal I/Eco RI
Lane 10 = Sal I/ Hind III
Lane 11 = Sal I/ Bam HI
Lane 12 = Sal I/ Xba I

The position of the arrow indicates the 3.3 Kbp fragment derived from pT20.

Figure 3-18: Southern blot of the T20 plasmid using ^{32}P labelled pUT 8 as the probe



A more sensitive test was undertaken to determine whether the protease produced by the recombinant pUT 8 was in fact the T20 protease. A modified method of Towbin *et al.*(1979) and Burnette (1981) and better known as the Western blot was used. Initially the proteins found in the intracellular and extracellular extracts of JM83 carrying pUT 8 were electrophoretically separated on a 12.5% polyacrylamide-SDS gel and stained with Coomassie Brilliant Blue (refer to Figure 3.20). Lanes 3 and 4 show those proteins occurring in both the intracellular and extracellular extracts of pUT 8/JM83. As controls, both the extracellular and intracellular proteins occurring in pUC 12/JM83 were separated as well and are shown in Lanes 5 and 6 respectively. Purified T20 extracellular protease is shown in Lane 7. None of the proteins occurring in the intracellular or extracellular extracts of JM83 containing pUC 12 alone or pUT 8 contain a protein of the same molecular weight as that of the T20 protease. These proteins were electroblotted on to a nitrocellulose membrane (refer to Figure 3.21) and were probed with polyclonal antibody raised against the extracellular protease produced by *P. fluorescens* T25 conjugated with IgG-GAR peroxidase using the Western blot technique (refer to section 2.10). As observed in Figure 3.21 the proteins were not bound by the T25 antibody. Consequently, it appears from this analysis that the pUT 8 recombinant does not encode the heat-stable protease.

This test also served to show that pT20 mutant which was unable to export the heat stable protease outside the cell, carried a protein in the intracellular extract that was the same molecular weight as the extracellular protein, refer to figure 3.20, lanes 2 and 7. This is unusual in that extracellular proteins do not lose their signal sequence until they are in transport to the outside of the cell and thus would be heavier than the extracellular mature protein.

**Figure 3-10: Immunoprecipitation of the
cell extracts of pUT 8 with purified T25 IgG**

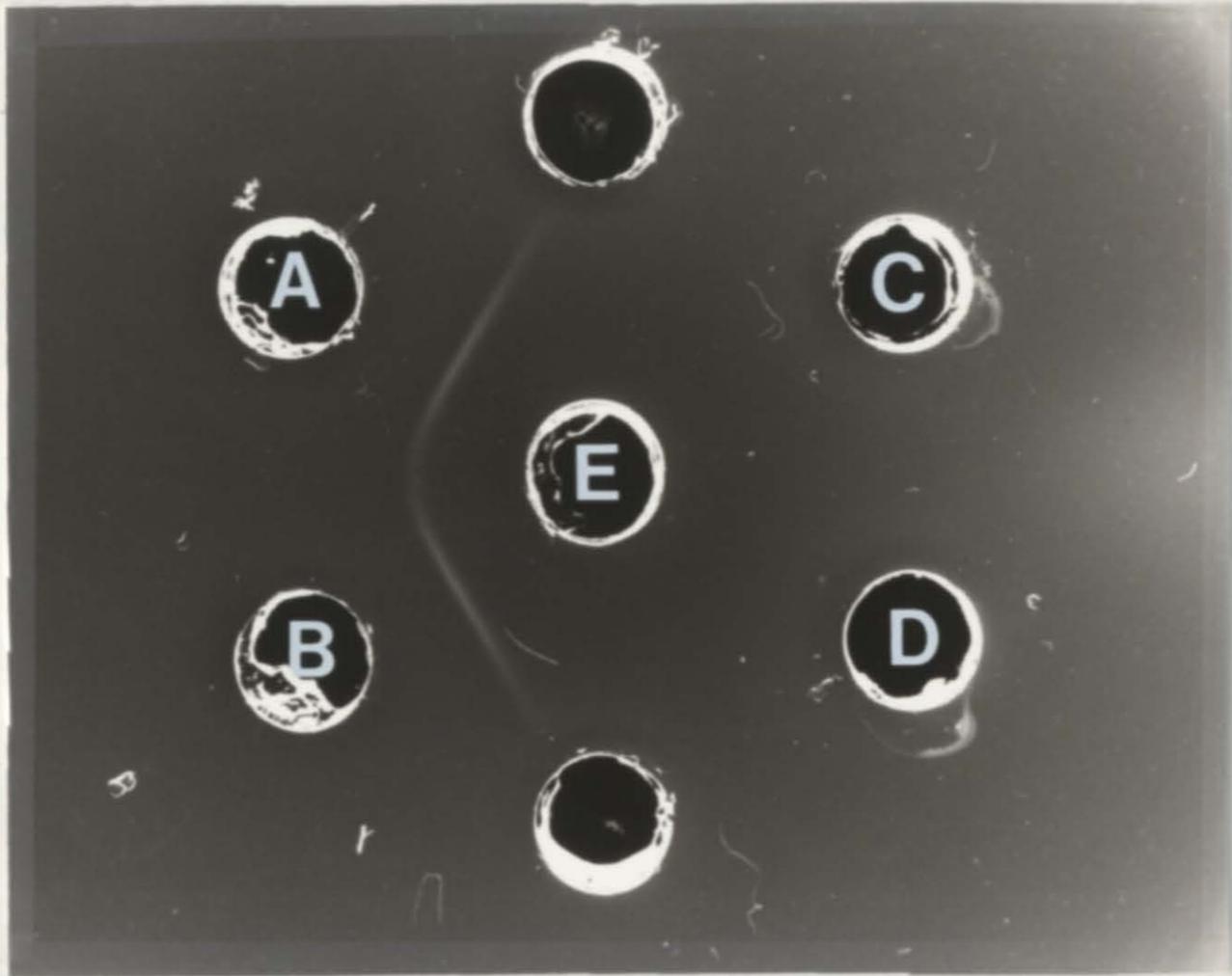


Figure 3.20 Proteins occurring in the cell extracts of pUT 8/JM83 and the P. fluorescens mutant, Type A

The proteins in the intracellular and the extracellular extracts were electrophoretically separated on a 12.5% SDS-polyacrylamide gel as described in section 2.9.2.

- Lane 1 = **P. fluorescens** extracellular extract, Type A
- Lane 2 = **P. fluorescens** intracellular extract, Type A
- Lane 3 = pUT 8 extracellular extract
- Lane 4 = pUT 8 intracellular extract
- Lane 5 = pUC 12 extracellular extract
- Lane 6 = pUC 12 intracellular extract
- Lane 7 = **P. fluorescens** T20 pure protease
- Lane 8 = molecular weight markers
 - a = myosin
 - b = B-galactosidase
 - c = phosphorylase B
 - d = bovine serum albumin
 - e = carbonic anhydrase

The arrows indicate the position of the T20 protease.

Figure 3-20: Proteins occurring in the extracts of pUT 8/JM83 and the *P. fluorescens* T20 mutant, Type A

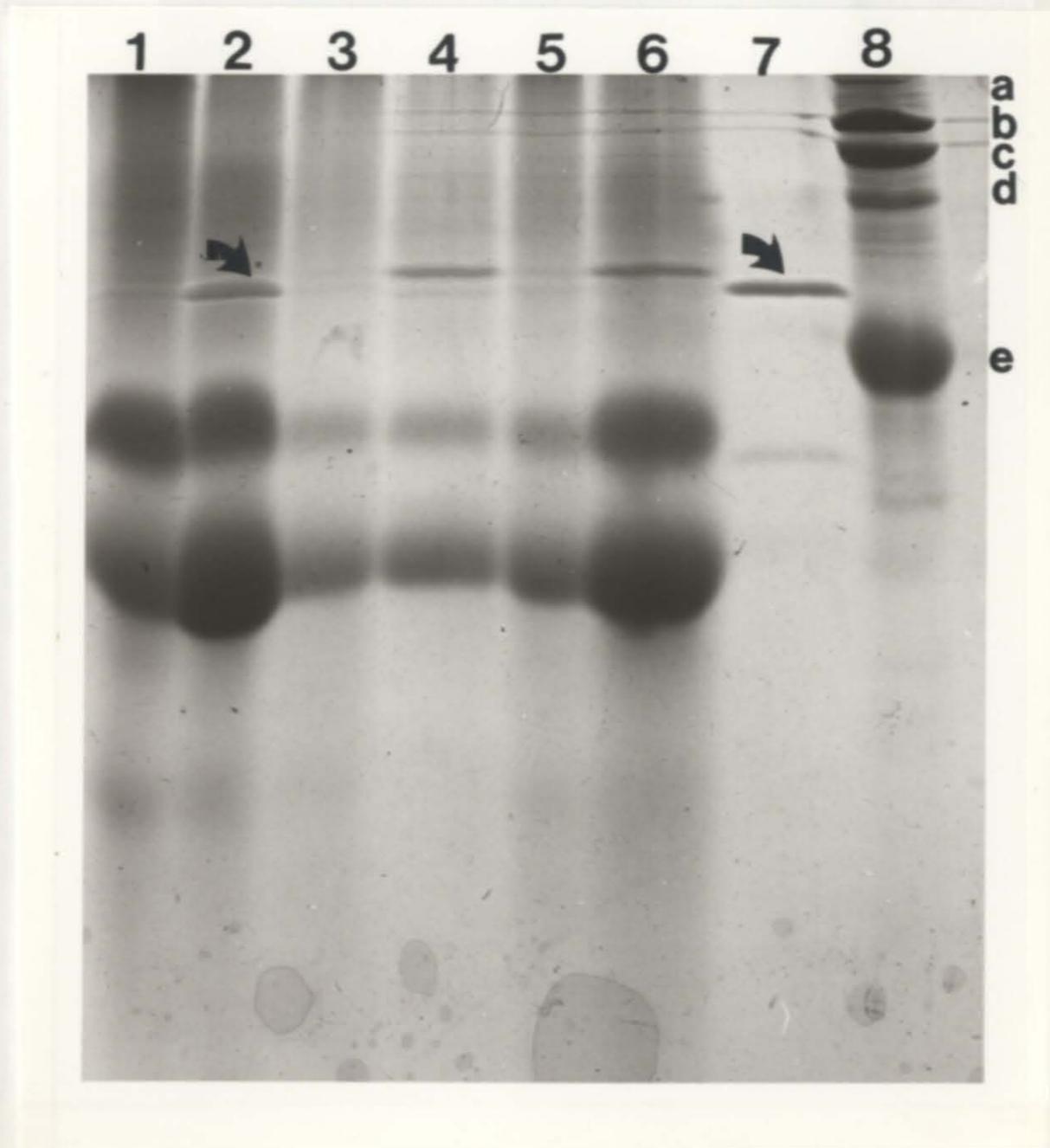


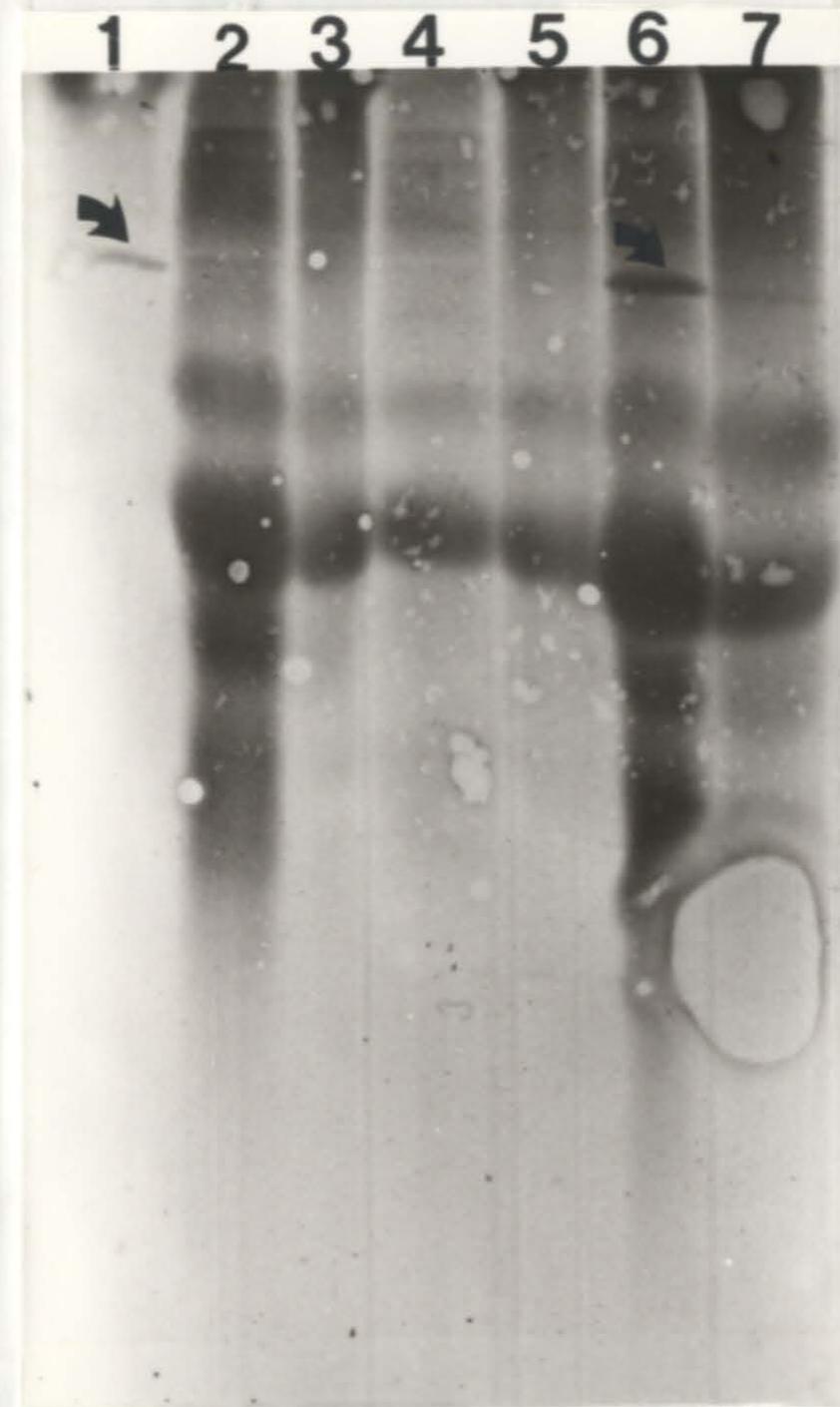
Figure 3.21 Western blot of the proteins occurring in the intracellular and the extracellular extracts of pUT 8 and the mutant T20 strain, T20A

Proteins occurring in both intracellular and extracellular extracts of pUT 8 and the mutant T20 strain which had been electrophoretically separated as previously described were electroblotted on a nitrocellulose membrane and probed with polyclonal antibodies raised against the *P. fluorescens* T25 protease as outlined in section 2.10. The arrow points to the heat-stable protease produced by T20 and its mutant strain, T20A.

- Lane 1 = *P. fluorescens* T20 pure protease
- Lane 2 = pUC 12/JM83 intracellular extract
- Lane 3 = pUC 12/JM83 extracellular extract
- Lane 4 = pUT 8 intracellular extract
- Lane 5 = pUT 8 extracellular extract
- Lane 6 = *P. fluorescens* mutant, Type A intracellular extract
- Lane 7 = *P. fluorescens* mutant, Type A extracellular extract

The arrows indicate the positions of the T20 protease.

Figure 3-21: Western blot of the proteins occurring in the intracellular and extracellular extracts of pUT 8 and the mutant strain, T20A



Through affinity chromatography it was possible to purify a fraction of *P. fluorescens* T20 (Figure 3-1) showing in the first peak, was not shown by HPLC analysis ensure that the protein SDS polyacrylamide gel indicating that this was The molecular weight calibration curve of the the ratio was very low molecular weight. The protein was shown to be 40,000 daltons. This is in agreement with work done by other investigators who found that a single heat stable protease is produced by *P. fluorescens* T20 (Patel et al., 1983). Also proteases of raw milk *Pseudomonas* fall within the molecular weight range of 40,000 daltons to 50,000 daltons (Harris-Egribaber et al., 1980).

While the mechanisms underlying the regulation and synthesis of extracellular proteases are the subject of ongoing research, the molecular origins of the heat-stable protease produced by raw milk *Pseudomonas* spp. has been largely ignored.

Chapter 4

Discussion

Through affinity column chromatography, using CBZ-L-phenylalanine TETA it was possible to purify the heat-stable protease from the crude extracellular extract of *P. fluorescens* T20 (Table 3.1). The elution profile of the chromatography (Figure 3.1) showed that two major peaks were eluted from the column. While the first peak was composed mostly of unbound protein, the second peak was shown by Hull's modified protease assay to contain the heat-stable protease. To ensure that the protease had been purified to homogeneity, it was run on a 7.5% SDS-polyacrylamide gel (Figure 3.2). A single protein band was observed indicating that only one heat-stable protease is produced by *P. fluorescens* T20. The molecular weight of the heat stable protease was estimated from a standard calibration curve of the log of the molecular weight of standard proteins versus the retention coefficient on Sephadex G 150 (Figure 3.3; Table 3.2). The molecular weight of the T20 protease was shown to be 40,000 daltons. This is in agreement with work done by other investigators who found that a single heat-stable protease is produced by *P. fluorescens* T20 (Patel *et al.*, 1983). Also proteases of raw milk *Pseudomonads* fall within the molecular weight range of 40,000 daltons to 50,000 daltons (Gebre-Egziabher *et al.*, 1980).

While the mechanisms underlying the regulation and synthesis of extracellular proteases are the subject of ongoing research, the molecular origins of the heat-stable protease produced by raw milk *Pseudomonas* spp. has been largely ignored.

Construction of a protease deficient mutant could facilitate the location of the sequence encoding the heat-stable protease produced by *P. fluorescens* T20. Using MNNG at a concentration which just permitted growth (3 $\mu\text{g}/\text{mL}$), mutants deficient in the export and /or synthesis of the heat-stable protease were produced. Both the intracellular and extracellular extracts of the mutant strains were assayed using Hull's modified protease assay for the presence of active protease; none of the mutant extracts showed any protease activity. Using IgG raised against the heat-stable protease of *P. fluorescens* T25 and which was known to cross-react with the heat-stable protease produced by the T20 strain (Patel *et al.*, 1983), two types of mutant strains appeared to have been generated. The mutant strain T20A, showed cross-reacting protein with the intracellular extract only (Figure 3.4) thus, it is able to express the protease, but appears unable to export it outside the cell. The mutant strain T20B showed no cross-reaction in either the intracellular or extracellular extracts (Figure 3.4), and therefore appears unable to export or express the heat-stable protease.

The proteins occurring in both the intracellular and extracellular extracts of the mutant strain T20A were run on a 12.5% polyacrylamide gel (Figure 3.20) as well as the pure T20 protease. While the T20A extracellular extract showed no protein band of the same molecular weight as the pure T20 protease, the intracellular extract did carry a protein which appeared to have the same molecular weight as the mature protein. A Western blot of the proteins occurring in the gel (Figure 3.21) showed that the protein which occurred in the intracellular extract of the mutant strain T20A did cross react with the probe and therefore was the T20 protease.

Proteins released to the extracellular environment by Gram-negative bacteria must cross both the inner and the outer cell membranes during their export. While much is now understood about how these proteins cross the cytoplasmic membrane, very little is known about how they are then translocated across the periplasmic space and across the outer membrane (Silhavy *et al.*, 1983). Most of

these proteins destined for export to the extracellular medium are synthesized with amino-terminal sequences known as signal sequences which are about 3 Kdal (Randall *et al.*, 1987). It would seem likely that one role of the signal sequence would be to mediate the interaction of precursors with proteins that are involved in the early steps in export (Emr and Silhavy, 1982). The protein isolated from the intracellular extract of the mutant strain T20A was observed to co-migrate with the mature T20 protein. It did not appear to have a slightly higher molecular weight than that of the mature T20 protein (Figure 3.20) and therefore did not appear to carry a signal sequence.

Of 32 prokaryotic signal sequences studied, it was found that even though lengths and amino acid sequences were extremely variable, all signal sequences were composed of three regions, a positively charged region of variable length (1 or 2 amino acids), followed by a stretch of 14 to 20 neutral, primarily hydrophobic amino acids, known as the hydrophobic core, and a stretch of approximately 6 amino acid residues after the hydrophobic core is predicted to form a reverse turn. This segment ends in a consensus processing site denoted AXB, where B is the last amino acid residue of the signal peptide and is alanine, glycine, or serine (Von Heijne, 1985).

The charge of the amino terminus of the signal peptide may be important in the coupling of translation to secretion across the inner membrane. Mutants in which the amino terminus of the signal peptide of the major outer membrane lipoprotein (Lpp) has a net negative charge show a 2 to 5 fold reduction in Lpp synthesis and slower rates of both its secretion and processing (Vlasuk *et al.*, 1983).

The hydrophobic core region of the signal peptide appears to be important in the association of the exported protein with the inner membrane as well as its secretion across the membrane. Both charged amino acids and deletions can disrupt the hydrophobicity of the core region in either a site specific way or by reducing the length below the minimal required for function (Randall *et al.*, 1987). Mutations that strongly block export of the maltose-binding (Mal E) have been

studied through suppressor analysis, where it has been observed that the length of the hydrophobic core region is critical in Mal E signal peptide function. Most mutations that restore secretion of Mal E lengthen the disrupted hydrophobic core region by insertion of additional hydrophobic amino acid residues through the extension of the amino terminal boundary of the core by a minimum of two amino acid residues (Bankaitis *et al.*, 1984). Also a recognition site within the core of the signal peptide may be disrupted through certain amino acid alterations thereby preventing the interaction of the signal sequence with the export machinery. Such a positional effect of signal sequence mutations within the Mal E signal sequence core region, block Mal E export (Bedouille *et al.*, 1980).

Mutations in the processing site appear to play a role in processing only. Mutations in this region can slow or eliminate processing but cannot affect the export of the mutant protein. The known processing site includes the region around the cleavage site that includes the last four amino acid residues of the signal sequence and includes the first two amino acid residues of the mature sequence. A mutation downstream from the signal peptidase cleavage site was observed to affect the cleavage reaction of the protein precursor with the signal peptidase but not the insertion into the membrane of the phage coat protein (Russel and Model, 1981). Further, when the signal peptide is not cleaved, it can have dramatic effects on the solubility of the secreted protein precursor (Koshland *et al.*, 1982).

The protein isolated from the T20A mutant strain co-migrated with the mature protein (Figure 3.22). It did not appear to carry a signal sequence. MNNG was the mutagenic agent used to induce mutants deficient in the synthesis and/or export of the heat-stable protease. This mutagen is known to form methylated bases in DNA both *in vivo* and *in vitro* (Walker, 1984). Treatment with such an agent produces a relatively high proportion (7%) of O⁶-methyl guanine (M⁶G) residues (Lawley and Orr, 1970), which are believed to be responsible for the high mutagenic activity of MNNG. The DNA repair pathway termed, the adaptive

response, appears to implicate M⁶G adducts in mutagenesis. One of the proteins induced during the adaptive response is methyl transferase, which removes methyl groups from M⁶G residues. Mutant strains that lack this enzyme show hypermutability when challenged with MNNG (Olson and Lindahl, 1980). The M⁶G residues in replicating DNA can pair with either cytosine or thymine, however in 29 of 30 MNNG induced mutations in the bacteriophage P22 *mnt* repressor gene, GC to AT base pair changes were induced (Lucchesi *et al.*, 1986).

Given the structure of the signal sequence it would not be very likely that a single base pair mutation in the mutant strain T20A could prevent the addition of the signal sequence to the precursor. Rather, it would more likely induce mutations through base pair changes in either of the three regions of the signal sequence, in the manner already described, which would strongly suppress or block the secretion of the protein across the inner membrane.

Since the protein extracted from the mutant strain appeared not to carry a signal sequence, it would seem to indicate that the protein had passed through the inner membrane and into the periplasmic space. Jenson *et al.* (1980) investigated the secretion of the extracellular protease, elastase, produced by *P. aeruginosa* and found that an inactive precursor of elastase with the same molecular weight as the mature protein was primarily located in the periplasmic space and believed that this indicated that elastase passed through the periplasm during its export and was activated through a change in conformation (Fecycz and Campbell, 1985). Therefore since signal peptides are structurally similar to those occurring on proteins destined for insertion in the inner and the outer membranes, it appears that mutations in the signal sequence affect translation, secretion and processing as a result of amino acids altered in the three regions of the leader peptide, and therefore are required to mediate the interaction of the precursor protein with proteins that are involved with the early stages of translocation.

The more distal steps in protein secretion are believed to depend on sequences

located in the mature protein. These sequences required for the secretion of the proteins are called topogenic sequences. Truncated proteins were studied to determine this. Chain terminating mutants which produced truncated versions of the maltose binding protein of *E. coli* (Mal E) that were 30% and 90% of the size of the wild type Mal E proteins. Both proteins were processed, but only the larger one was found in the periplasm while protease protection experiments showed that a portion of the smaller fragment was exterior to the cytoplasmic membrane (Ito and Beckwith, 1981). Topogenic sequences have been located in the mature portion of the outer membrane protein of *E. coli*, the lambda receptor (Lam B). There is a sequence within the first 49 residues of the mature protein which allows it to achieve an outer membrane location (Benson *et al.*, 1984).

Assuming that the periplasmic contents of the mutant strain T20A had been isolated without contaminating cellular debris, then it appears that the protein was successfully translocated through the inner membrane and, cleaved of its signal sequence, either co- or post-translationally, it remained in the periplasmic space. A mechanism must exist which differentiates between soluble proteins destined for the periplasm and those destined for the extracellular medium. Since the heat-stable protease normally found in the extracellular medium of the wild type cells was isolated apparently in the periplasm of T20A, it would suggest that either the periplasm is normally part of the export route or that the protein has been diverted there as a result of defects in the extracellular export mechanism.

The simplest possible pathway for extracellular excretion would involve recognition of the protein by a receptor at zones of adhesion and co- or post-translational movement across the inner and outer membranes simultaneously. Lory *et al.* (1983), observed that export of exotoxin A in *P. aeruginosa* occurred as rapidly as the protein was synthesized and therefore would appear to support such a model. A less direct route for protein export would require transfer of the protein across the inner membrane, sorting either on the outside surface of the inner membrane or the periplasm, and transfer across the outer membrane. The

fate of cholera toxin in *Vibrio cholerae* and heat-labile enterotoxin in *E. coli* cells appears to support such a two-step model. While cholera toxin is found in the supernatants of *Vibrio cholerae* cultures, it remains within cells of cultures of *E. coli* containing the gene for the cholera toxin (Pearson and Mekalanos, 1982). Further the heat-labile enterotoxin which is a periplasmic protein in *E. coli* is exported to the extracellular medium by *V. cholerae* strains that contain enterotoxin plasmid (Neill *et al.*, 1983). While it could be argued that the distribution of proteins synthesized in foreign hosts may be abnormal, the extensive genetic and structural homology between the heat-labile toxin and the cholera toxin decrease this possibility (Pearson and Mekalanos, 1982). Further, it has been suggested that proteins destined for export remain associated with the outside of the inner membrane after the translocation and processing and that they are somehow moved to specific export sites, ie zones of adhesion (Lory *et al.*, 1983).

It is possible that the protein extraction from the intracellular space of the mutant strain T20B successfully traversed the inner membrane and had its signal sequence cleaved and was isolated from the periplasm. The mutation induced by MNNG would then appear to have been located, not in the signal sequence, but rather in the export machinery that is more distally located in the export process, ie. if the export mechanism is one such as that proposed by Lory for *P. aeruginosa*, then instead of being passed through zones of adhesion, the protein was diverted at some point through to the periplasm where it remained trapped and in a precursor conformation that precluded or prevented it from being active as its inactivity was illustrated by Hull's modified protease assay. If however the protein is normally secreted in the two-step process proposed for *Vibrio*, by Pearson and Mekalanos (1982), then the periplasm is part of the normal export route and therefore MNNG may have caused a mutation as it traversed the outer membrane assuming that the tertiary structure of the protein was not affected.

The results in this study make it very difficult to actually choose one of the two

proposed routes of export for the heat-stable protein, due to the method used to pinpoint its location within the cell. The methods used to identify the location of the protein are inherently problematic, since, experimental methods validated for the normal and permanent components of a given fraction may in fact, be inappropriate for defining the location of abnormal or transient components in the export pathway, ie. when bacteria are in an abnormal physiological state where export is blocked by a mutation in a component of the export apparatus. This study utilized periplasmic shock and sonication to release the intracellular material followed by centrifugation at 15,000 g for 20 minutes. In fact, this would not have been enough force to remove all fragments of the outer membrane or inner membrane and those proteins associated with them. As observed in Figure 3.22, the intracellular fractions contained other proteins which were the same molecular weight as those derived from the extracellular extracts. They may have been embedded in either the outer or inner cell membranes which were not removed during centrifugation. Ideally, cell contents should have been centrifuged at 10^5 g for 2 hours to remove membranes and their associated proteins from the soluble fraction of cell lysate. Subsequently, it is difficult to interpret the results described here and correctly determine whether the protein extracted from the mutant T20A strain was in the periplasmic space or whether it was attached to the inner or outer membranes. To establish this it would require that several experimental methods be used, all leading to the same conclusion: for example, isopycnic density gradient centrifugation in sucrose (Miura and Mizushima, 1968), differential solubilization of protein in non-ionic detergents (Filip *et al.*, 1973), or immunological microscopy using colloidal gold (Horisberger, 1979) or pulse-chase experiments.

A second mutant strain was generated, T20B, which was shown by immunoprecipitation not to produce the heat-stable protease in either the intracellular or the extracellular extracts (Figure 3.4). Thus the mutant strain, T20B, did not appear to express the heat-stable protease. As illustrated in Figure 3.5 *P. fluorescens* T20 was shown to contain a plasmid. The mutant strains T20A

and T20B were also examined for the presence of a plasmid. T20A was shown to carry a plasmid, which co-migrated with the plasmid occurring in T20, while T20B did not carry a plasmid. From this it appeared that MNNG had in fact cured the strain T20 of its plasmid. As previously described, MNNG produces a relatively high proportion of O⁶-methyl guanine residues (Lawley and Orr, 1970). In replicating DNA, M⁶G residues can pair with either cytosine or thymine (Loveless, 1969). In fact, 95% of all substitutions in the *lacI* gene of *E. coli* are GC to AT transitions (Coulondre and Miller, 1977). If such an event occurred which interfered at any point of replication i.e. either within the replication origin itself, its promoter or any of those regions encoding proteins involved in replication would inhibit DNA replication. Such methylation at the origin of replication are apparently essential for Ori C function. GC to AT transition would prevent or at least reduce the probability of replication initialization (Smith *et al.*, 1985). This would be particularly significant if the plasmid had a low copy number. As well, DNA A protein which appears to act both before and during the essential transcription event and which binds to the origin in the form of a multimeric complex by interaction with the DNA A boxes, is considered a key protein in the initiation of replication (Altung and Hansen, 1983). If such O⁶-methylations created nonsense reading frames for the DNA A protein or the DNA A boxes, such that DNA A protein synthesis was blocked DNA replication would cease (Schaus *et al.*, 1981). From this it appears that the plasmid occurring in T20 may encode the heat-stable protease produced by *P. fluorescens* T20, since the mutant strain T20B, does not carry a plasmid, nor does it produce the heat-stable protease.

To confirm that the strain T20B had been cured of its plasmid, it would be prudent to extract the chromosomal DNA from *P. fluorescens* T20, digest it with a restriction endonuclease and probe it in Southern hybridization experiments with radiolabelled T20 plasmid, to ensure that there were no homologous sequences on the chromosome which might encode the heat-stable protease. Also alternative means of curing the strain of its plasmid could be employed, such as

heat-shock or treatment with rifampin or the introduction of a *Pseudomonas* incompatibility plasmid which does not encode proteases.

Since a number of *Pseudomonas* species are known to carry plasmids which mediate resistance to a variety of antibiotics, the antibiotic resistance spectra of the wild type T20 and its mutant strains, T20A and T20B was investigated. In fact, the pattern of resistance for those antibiotics outlined in Table 3.3, remained the same for all except the mutant strains were sensitive to chloramphenicol, while the parent strain was not. Since T20A carried a plasmid and was sensitive to chloramphenicol, this would suggest that chromosomally encoded chloramphenicol acetyltransferase (CT) is produced by T20. However *Pseudomonas* is known to have plasmid encoded CT (Nitzan and Rushansky, 1981). To determine whether the T20 plasmid does encode CT the T20 plasmid could be transformed into a host sensitive to chloramphenicol. Alternatively, the chromosome could be probed in Southern hybridization experiments with a plasmid encoding CT. Should homologous regions occur, those regions could be probed with the T20 plasmid using the method of Southern (1975). Only then could one be certain that the plasmid encoded CT as well as the chromosome.

Plasmid encoded degradation of simple organic as well as hydrocarbon and synthetic compounds have been well characterized in *Pseudomonas* spp. For example the CAM plasmid occurring in *P. putida* controls camphor oxidation (Rheinwald *et al.*, 1973). Also in *P. putida*, another plasmid, NIC, codes for nicotine catabolism (Thacker and Gunsalus, 1979). There are in fact a number of pseudomonads which carry what have come to be called catabolic or metabolic plasmids which break down a number of compounds into utilizable energy sources through a multistep reaction sequence.

A plasmid was found in *P. fluorescens* T20 (Figure 3.5) and based on the results obtained in sections 3.31 to 3.34 previously discussed, it appeared that the heat-stable protease was encoded by that plasmid. Using a series of single and double restriction endonuclease digestions (Figures 3.6-3.8), it was possible to estimate

that the plasmid was 44.06 Kbp. This is small in comparison to other plasmids which have been extensively studied in other strains of *P. fluorescens* and other *Pseudomonas* spp. One of the smallest is pEG, which occurs in *P. fluorescens*. It is 37 Kbp and aids in styrene catabolism (Bestetti *et al.*, 1984). One of the largest plasmids isolated is OCT (500 kbp) which aids in octane,decane metabolism (Shapiro *et al.*, 1984). To facilitate the construction of a gene library, a physical map of the T20 plasmid was constructed (Figure 3.9). It was determined that the plasmid carried a number of sites for each of *Sal* I, *Pst* I, *Xba* I, *Hind* III, *Bam* HI , *Eco* RI, and *Sac* I (Figures 3.6-3.8). The orientation of the sites was determined from the molecular weights of the fragments (Tables 3.4-3.6) using the MAPC program. The proposed map (Figure 3.9), is only a partial map. Those endonucleases listed were used because they were readily available and provided a range of fragments that were suitable for cloning and transformation since the many of the fragments generated were less than 5 Kbp.

While mapping using MAPC is a fast and relatively easy means to interpret the restriction endonuclease data, only single and multiple digestions were used to obtain the fragment sizes. MAPC gives a total of eight possible solutions which fall within the error limits selected ie. 0.01 to 0.05. Where digestions appear incomplete as with *Xba* I and *Sac* I, it introduces more error into the program. Often other techniques are used in concert to determine a detailed physical map. They include sequential digestion, here double digestions are performed, and fragments that disappear after the second digestion contain sites for the second enzyme, new bands represent unique double digestion products. Using the molecular weights of the resultant fragments, a physical map can then be constructed (Jorgensen *et al.*, 1979).

A more accurate, though more time consuming method involves digestion with an exonuclease such as *Bal*31 (Legerski *et al.*, 1978). This exonuclease degrades both 3' and 5' strands of linear DNA molecules in a progressive manner. The reaction can be stopped at different time intervals, the DNA extracted with

phenol/ether to remove the nuclease, and redigested with the endonuclease of interest. The digests are electrophoresed along with a control (DNA not treated with *Bal31*, but digested with a selected endonuclease). The order in which fragments disappear on the gel indicates the order of fragments from the ends of the DNA molecules. Thus the fragments are correctly ordered.

A *Sal* I fragment of pT20 was successfully cloned into a cloning vector pUC12 (Figure 3.11, Lane 9). The fragment was found to be 3.3Kbp (Figure 3.12) and a partial physical map was proposed (Figure 3.15) based on both the single and double digestion products (Figures 3.13 and 3.14). This endonuclease was selected because it gave a number of small fragments (ie less than 5Kbp). Size of the fragments to be cloned is a limiting factor in the transformation efficiency of the recombinant since efficiency of transformation decreases with increasing plasmid size. Ideally the recombinant to be cloned should be 2-10 Kbp (Collins and Hohn, 1978). The recombinants were transformed into *E. coli* DHI. Using pKT230, a broad host range vector, to check the competence of the cells, the frequency of transformation was 3.2×10^6 transformants per μg of DNA. The cloning vector pUC 12 was chosen to clone the pT20 *Sal* I fragments because it carried a resistance to ampicillin which would allow detection of a transformant. To locate that fragment which encoded the heat-stable protease the transformants were also plated out onto a LA plate incorporated with both ampicillin and 2% milk powder. Those colonies which produced a zone of proteolysis were selected for further study (Figure 3.10). A single fragment was found to occur in only one of the 12 colonies selected (Figure 3.11). This fragment was proven to have been derived from pT20 using Southern hybridization. While the probe, radiolabelled pT20 hybridized with the fragment cloned, it did not hybridize with the vector, pUC12, also indicating that pT20 did not share any homologous regions with the vector (Figure 3.16). To detect whether the heat-stable protease was actually being expressed by the recombinant carrying the pT20 *Sal* I region, pUT 8, the intracellular and extracellular extracts were analysed for cross-reacting inactive protein with IgG raised against the heat-stable protease produced by *P.*

if the protein was expressed was also utilized. The Western is a more time consuming and costly method but it is known to be more sensitive than the immunoprecipitation method previously described (Towbin *et al.*, 1979) This method as well indicated that pUT 8 did not express the heat-stable protease since there was no hybridization with the probe (Figure 3.21). In order to ensure that the Western was capable of detecting small quantities another control should have been included ie a protein concentration curve which would have shown the exact limits of detection using the procedure outlined in section 2.10.

Only one of twelve colonies selected for further study carried a *Sal* I fragment, while all showed zones of proteolysis. Originally a control, pUC 12 transformed *E. coli* DHI, had been incubated at the optimum temperature of incubation for *E. coli*, 37°C for 72 hours (Figure 3.10). The resultant growth did not produce a zone of proteolysis. However, when *E. coli* was transformed with pUT 8, the temperature of incubation was lowered to the optimum temperature for *P. fluorescens*, 25° since it was believed that the increased incubation temperature might possibly inactivate the heat-stable protease or inhibit its expression. However at a lower temperature the *E. coli* strain probably began to produce enzymes not normally produced at its optimum temperature and this would have been responsible for the limited zones of proteolysis surrounding the transformed colonies. This seemed an unlikely scenario since *E. coli* strains have not been observed to produce extracellular proteases, and do not appear to be equipped with a mechanism for such extracellular transport as are the *Pseudomonas* spp. However this does not preclude the possibility of proteases bound in the outer membrane of the *E. coli* strain that become active at lower temperatures. On closer observation of *E. coli* DHI transformed with pUC12, limited zones of proteolysis were produced around the colonies when incubated at 25°C and the zones became much broader upon storage at 4°, though no doubt, some of this is attributable to some cell death over time as well as to the production of other enzymes that may be bound in the outer membrane and may become active at lower temperatures.

enzymes that may be bound in the outer membrane and may become active at lower temperatures.

The vector, pUC 12, is a complementary vector, in that, it complements deletions in the amino terminal end of the lac Z gene since the vector carries the Lac Z gene which has deletions in the carboxy terminal end. The truncated peptides produced by both, aggregate and restore β -galactodidase activity. *E. coli* JM83 carries such a deletion. When pUC 12/JM83 is plated onto media containing X-gal, the resultant color reaction gives rise to blue colonies. If pUC 12 contains an insert its ability to produce the complementary truncated peptide is inhibited and white colonies result. In an attempt to clone other fragments derived from *Sal* I as well as fragments derived from digestions using other endonucleases such as *Pst* I and *Eco* RI this strain was used. This strain was not observed to produce a zone of proteolysis around the colonies when transformed with pUC 12 when incubated at lower temperatures. Thus it provided a third phenotypic character by which a clone could be detected.

While the recombinant pUT 8 was successfully transformed into JM83, other attempts to clone other fragments generated using those endonucleases listed above were not successful. To minimize recircularization of the vector, alkaline phosphatase was used to remove the 5' phosphate, effectively inhibiting the DNA ligases. Since the DNA fragment to be cloned is not exposed to the enzyme it has 5' ends and can be ligated to the ends of the plasmid vector. This method as well proved unsuccessful, since transformants found only carried pUC 12.

To successfully create a library and possibly locate that region encoding the heat-stable protease, it appears necessary to use broad host range, high copy number cloning vectors such as RSF1010 (Barth and Grinter, 1974) which are propagated stably in pseudomonads and other Gram-negative bacteria. This together with a commercially available strain of *Pseudomonas* such as PAO110, which does not carry a plasmid or produce a heat-stable protease would probably allow the cloning and expression of the fragment encoding the heat-stable protease.

Chapter 5

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Appendix I

SDS Gel Preparation

Materials:

Solution A:	Acrylamide	30.0 g
	N,N'methylenebisacrylamide	0.8 g
	Distilled water	100 mL
Solution B(4X):	Tris base	18.2 g
	6N HCl	20.0 mL
	Adjust pH to 8.8	
	10% SDS	4.0 mL
	Bring volume to 100 mL with distilled water.	
Solution C(4X):	Tris base	6.06 g
	Adjust pH to 6.8 with 6N HCl	
	10% SDS	4.0 mL
	Bring volume to 100 mL with distilled water.	
Solution D:	Ammonium persulfate	10.0 g
	Distilled water	100 mL
Running Buffer:	Tris base	12 g
	Glycine	57.6 g
	Distilled water	1000 mL
	Adjust pH to 8.3. Dilute 1:4 with distilled water before use.	
Sample Buffer:	Glycerol	10.0 mL
	B-mercaptoethanol	5.0 mL
	10% SDS	30.0 mL
	Solution B (4X)	12.5 mL
	Adjust volume to 100 mL with distilled water.	
Running gel:	Distilled water	16.6 mL
	Solution B	10.0 mL
	Solution A	13.3 mL
	Solution D	200 uL
	TEMED	10 uL
Stacking gel:	Distilled water	3.25 mL
	Solution C(4X)	1.25 mL
	Solution A	500 uL
	Solution D	150 uL
	TEMED	5 uL

Appendix I

Protocol:

Gel tubes were soaked overnight in chromic acid. They were then rinsed three times in distilled water. The tubes were soaked in 1% Photoflo and were dried in an oven at 40°C. The tubes were marked 12.5 cm from the bottom and were filled with running gel to that mark. They were then layered with 0.1% SDS and allowed to solidify. The stacking gel was then prepared and approximately 10 drops were layered on top of the running gel. The gels were then used within 24 hours.

Appendix II

SDS Slab Gel Preparation

Materials:

Solution A:	Acrylamide	30.0 g
	N,N'methylenebisacrylamide	0.8 g
	Distilled water	100 g
Solution B: (4X)	Tris base	18.2 g
	6N HCl	20.0 mL
	Adjust pH to 8.8	
	10% SDS	4.0 mL
	Bring volume to 100 mL with distilled water.	
Solution C: (4X)	Tris base	6.06 g
	Adjust pH to 6.8 with 6N HCl	
	10 % SDS	4.0 mL
	Bring volume to 100 mL with distilled water.	
Solution D:	Ammonium persulfate	1.0 mg
	Distilled water	100 mL
Running Buffer:	Tris base	12.0 g
	Glycine	57.6 g
	Distilled water	1000 mL
	Adjust pH to 8.3. Dilute 1:4 with distilled water before use.	
Sample buffer:	Glycerol	10.0 mL
	B-mercaptoethanol	5.0 mL
	10 % SDS	30.0 mL
	Solution B (4X)	12.5 mL
	Adjust volume to 100 mL with distilled water.	
Running gel:	Solution A	15.0 mL
	Distilled water	11.4 mL
	Solution C (4X)	9.0 mL
	Mix and de-gas.	
	Solution D	144 uL
	TEMED	17 uL
Stacking gel:	Distilled water	6.0 mL
	Solution B (4X)	2.5 mL
	Solution A	1.5 mL
	Mix and de-gas	
	Solution D	100 uL
	TEMED	10 uL

Appendix II**Protocol:**

Prepare glass plates by soaking in chromic acid overnight and following this with three rinses in distilled water. The plates were then soaked in 1% photoflo for 10 minutes and allowed to air dry. The running gel was poured between the plates, which had been set up in a Huffer apparatus, up to the 12 cm mark. The gel was overlaid with butanol and allowed to set. After this, the gel was rinsed with running buffer and blotted with filter paper. Next the stacking gel was added and a comb was inserted and the gel was allowed to set. Protein was loaded in a total volume of 200 μ L of sample buffer less 5 μ L of 0.1% bromophenol blue and volume of protein.

Appendix III**Molecular Hybridization Reagents**

- **A. Standard Saline Citrate:** 3.0 M Sodium Chloride
(20X) 0.3 M Sodium Citrate

- B. Denhardt's Reagent:** 1% Ficoll
(50X) 1% Polyvinylprolidone
1% Bovine Serum Albumin
distilled water
Filter and store at -20°C.

