



B. AMYLOLIQUEFACIENS ALKALINE PROTEASE SYNTHESIS:

GENE CLONING

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by

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SUMMARY

1. The work described in this thesis was directed towards isolation of the gene for the extracellular alkaline protease of *B. amyloliquefaciens*, to enable a study of the production of this enzyme, at the molecular level, to be undertaken.
2. RNA from *B. amyloliquefaciens* was isolated, and translated in a cell-free system derived from *E. coli*. An antiserum prepared to the extracellular alkaline protease was used to identify translational products related to this enzyme.
3. The mRNA for these products was partially purified by fractionation of total RNA on sucrose-formamide gradients followed by agarose-methylmercury gels.
4. Random primed cDNA synthesised to the partially purified mRNA preparation was used to screen a genomal library of *B. amyloliquefaciens* DNA. A clone was isolated, which, by using the technique of hybrid-selection-translation, appeared to correspond to a major translational product shown by immunoprecipitation to be related to the alkaline protease. DNA sequence analysis did not confirm the identity of this putative alkaline protease clone. The possibility exists that this clone may be related to the extracellular neutral protease of *B. amyloliquefaciens*.
5. Using RNA from *B. amyloliquefaciens* as template, and synthetic DNA oligomers specific for the alkaline protease gene as primers, extended probes were synthesised and used to screen a colony library of *B. amyloliquefaciens* DNA. This resulted in the isolation of a clone, designated pBAP1,

which was confirmed by DNA sequence analysis to contain the gene for the extracellular alkaline protease of *B. amyloliquefaciens*.

6. The cloned alkaline protease gene was used as a probe to examine mRNA production by hybridisation analysis. Preliminary experiments are supportive of the concept of a reserve pool of mRNA for this enzyme.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, it contains no material previously published or written except where due reference is made in the text.

MICHAEL JAMES BAWDEN



ABBREVIATIONS

The abbreviations used in this thesis are acceptable to the Journal of Molecular Biology or are defined in the text.

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CHAPTER ONE

INTRODUCTION



## GENERAL INTRODUCTION

The work presented in this thesis concerns the synthesis of extracellular protease by *B. amyloliquefaciens*. This organism produces several extracellular enzymes, the synthesis of which have been studied extensively in this laboratory. Of particular interest in this work has been a study of the production of extracellular protease, which shows an unusual response to inhibitors of transcription. As well as providing a model system for the study of exoenzyme production in bacteria, a novel control situation is apparent in the production of extracellular protease by *B. amyloliquefaciens*. The central aim of the work presented here has been to examine this phenomenon at the molecular level, as will be discussed further, later in this chapter.

### 1.1 INTRODUCTION

Bacterial extracellular enzymes are largely degradative and their most obvious role seems to be in hydrolysis of environmental macromolecules for utilisation by the cell.

(Mandelstam, 1969; Engelking & Seidler, 1974). In this way these enzymes probably fulfil the same role as the various digestive enzymes secreted by organs such as the pancreas. In many species of *Bacillus*, secretory proteases may also play a role in the sporulation process, as evidenced by the finding that these enzymes are produced in response to a developmental signal which directs the bacteria to sporulate. (Hoch, 1976, Priest, 1977).

The problem arises, in both prokaryotes and eukaryotes,

of how in many cases, potentially lethal enzymes are synthesised and secreted with impunity across biological membranes. This problem is encompassed in the broader question, fundamental in the function of all living cells, of how hydrophilic proteins, whatever their final destination and function, are translocated across hydrophobic membranes.

## 1.2 TRANSPORT OF PROTEINS ACROSS BIOLOGICAL MEMBRANES

It has become clear in the past ten years or so, that most transported proteins of both prokaryotes and eukaryotes are synthesised with amino-terminal "signal" peptides which play an important part in the transport process. Fundamental problems, which have been the focus of intensive research in recent years include: the role of the signal peptide in the transport process; the involvement of other proteins in the mechanism of transport; whether transport occurs as the protein is elongated, or after synthesis, involving the transfer of entire domains; the location and timing of processing of the transported protein by the signal peptidase; the location of the synthesis of proteins destined for transport (i.e. whether this occurs on membrane-associated or free polysomes) and the importance of primary and secondary structures (both in the signal peptides and in the processed proteins) in determining the final location and topology of secretory and membrane-associated proteins.

While it has been established that prokaryotes and eukaryotes share common features in the process of protein transport, many details of the mechanisms of protein trans-

location remain to be elucidated. In eukaryotes, secretory proteins are largely synthesised on polysomes associated with membranes, and examples also exist in bacteria. A considerable body of evidence also exists for the synthesis of secretory proteins on free polysomes.

The very large body of work in this field, in both eukaryotes and prokaryotes, has been the subject of extensive reviews in recent years, [Glenn, (1976); Wickner, (1979); Inouye & Halegoua, (1980); Davis & Tai, (1980); Blobel, (1980); Emr *et al.*, (1980); Wickner, (1980); Kreil, (1981); Michaelis & Beckwith (1982); Silhavy *et al.*, (1983)].

This material will not be covered again here except to consider briefly four models emerging from this work which attempt to explain the mechanism of protein transfer across biological membranes. These models may be considered to fall into two categories. First, three models in which the polypeptide chain is seen to be extruded in a linear form, generally as it is elongated. These models are the signal hypothesis (Blobel & Dobberstein, 1975), the helical hairpin hypothesis (Engelman & Steitz, 1981) and the direct transfer model (Von Heijne & Blomberg, 1979). In these models emphasis is placed on the importance of the primary structure of proteins in the transport process. In the second category, represented by the membrane-trigger hypothesis (Wickner, 1979), emphasis is placed on the secondary structure of proteins, with the transfer of entire domains across the membrane after synthesis. It seems possible that the real situation for protein transport resides in the still somewhat grey area somewhere between these extremes.

In the signal hypothesis (Blobel & Dobberstein, 1975),

originally proposed to explain the co-translational extrusion of proteins across the endo-plasmic reticulum of eukaryotic cells, the signal peptide of the nascent secretory protein was seen to direct the binding of the ribosome to a proteinaceous pore in the membrane which facilitates passage of the nascent chain through the bilayer. This model can be modified to account for post-translational modes of transport (Blobel, 1980), but requires transfer of the protein in an extended form. The SRP (signal recognition particle) a complex of six proteins and a 7S RNA species, has recently been shown to play a part in the translocation process (Walter & Blobel, 1983). The SRP is proposed to bind to the signal sequence of a nascent secretory protein, blocking translation until the appropriate membrane is encountered. Binding of the signal/SRP complex to a membrane integrated receptor, termed the "docking protein", results in release of elongation arrest and the formation of a hydrophilic, transmembrane pore through which the secretory protein is co-translationally extruded. The signal sequence is cleaved by the signal peptidase which is associated with the pore complex.

In the helical hairpin hypothesis (Engelman & Steitz, 1981), it is proposed that translation by cytoplasmic ribosomes results in the synthesis of the initial segment of a secretory protein which spontaneously partitions into the membrane in the form of a hairpin containing two helices, one of which is the hydrophobic signal sequence. The rest of the secreted protein sequence forms a polar helix as it passes through the membrane as protein synthesis continues. Cleavage of the signal peptide results in the release of the mature protein on the extra-

cytoplasmic side of the membrane. This process is spontaneous and depends on the distribution of polar and non-polar sequences in the polypeptide, and by necessity is co-translational to prevent folding of domains within the cytoplasm.

The direct transfer model (Von Heijne & Blomberg, 1979) proposes that after the initiation of translation by a cytoplasmic ribosome, the initial segment of a secretory protein partitions into the membrane as a loop containing two  $\alpha$ -helices. The ribosome binds to an integral membrane protein and continued translation results in extrusion of the secretory protein through the membrane. The mature protein is released on the extra-cytoplasmic side of the membrane after cleavage of the signal region and the ribosome dissociates from its binding site. This model suggests, that provided the ribosome is tightly associated with the membrane, the energy of elongation would be sufficient to drive the chain directly through the bilayer.

An alternative to the above three models is the "membrane trigger" hypothesis put forward by Wickner (1979), which emphasises the role of protein conformation in the transport process. This model proposes that the interaction of a protein with the membrane triggers its folding into a conformation that spans the bilayer or is integrally associated with it. This spontaneous process may involve the rearrangement of a protein's hydrophobic and hydrophilic domains. The role of the signal peptide is to activate a protein for membrane assembly by altering its folding pathway. The signal peptide facilitates protein translocation by promoting the proper folding of the protein as it encounters the bilayer, the need for specific



ribosome-membrane interactions is alleviated, and processing occurs after translation is complete. This model involves the movement of entire domains of a protein through the membrane, and unlike the other models, which in general rely on co-translational extrusion of nascent chains through the membrane, while not obligatorily post-translational, encompasses this mode of transfer.

There is increasing evidence that many proteins can be transported by either co- or post-translation mechanisms (Silhavy *et al.*, 1983), ie, that these two modes of transfer are not mutually exclusive for a particular protein, and that there may not be any fundamental difference between post- and co-translational mechanisms of transport (Randall, 1983).

In summary then, it can be said that the detailed mechanism of transport of proteins across membranes has not yet been elucidated. Extracellular enzyme production by *B. amyloliquefaciens* has been used as a model system in this laboratory to study this process in bacteria, and this work is discussed below.

#### EXTRACELLULAR ENZYME PRODUCTION BY *B. AMYLOLIQUEFACIENS*

Exoenzyme production by *B. amyloliquefaciens* and transport of these proteins across the membrane has been studied in this laboratory over many years. This organism secretes into the external medium large amounts of  $\alpha$ -amylase, ribonuclease, and protease, of which there are two major species, an alkaline protease and a neutral protease. As a system for studying

exoenzyme production it has the advantage that washed cells, free of accumulated extracellular enzymes, can actively produce these enzymes under conditions where the cells are not growing.

Work by Both *et al.* (1971, 1972) indicated that washed cells of *B. amyloliquefaciens* harvested in late log phase of growth contained an apparent pool of protease mRNA sufficiently large to sustain rapid protease synthesis for about sixty minutes in the presence of rifampicin or actinomycin D. The mRNA for intracellular proteins decayed as expected within a few minutes of treatment with the antibiotics (as measured by ability to incorporate amino acids). The synthesis of protease was shown to involve *de novo* synthesis of protein in two important control experiments. Firstly, chloramphenicol prevented it and secondly, the protease released after 20 min incubation was totally labelled with  $^{14}\text{C}$ -leucine indicating *de novo* synthesis of the enzyme.

Gould, May & Elliott (1973) and Love (1981) showed the existence of qualitatively similar pools for  $\alpha$ -amylase and ribonuclease though of a smaller apparent size. Sanders and May (1975) showed that *B. amyloliquefaciens* extracellular enzymes emerge from the protoplast in a trypsin-sensitive configuration implying that either a nascent form is extruded or that the enzyme is secreted in a form different from the final form which is insensitive to trypsin. The antibiotic cerulenin, which prevents fatty acid synthesis in a wide range of organisms has been shown by Paton *et al.* (1980), in an interesting study, to selectively inhibit protein secretion by *B. amyloliquefaciens*.

The synthesis of extracellular protease by *B. amyloliquefaciens* is a system whose response to inhibitors of transcription is very unusual when compared to other prokaryote systems. This has been of central interest in recent work in this laboratory and is the focus of the work to be described in this thesis.

Protease production is subject to amino acid repression, and as mentioned, involves *de novo* synthesis of the enzyme (Both *et al.*, 1972; May & Elliott, 1968). O'Connor (1978) demonstrated that incubation in a medium with high levels of amino acids (repressive) for 75 min exhausts the capacity of cells of *B. amyloliquefaciens* to produce extracellular protease in the presence of rifampicin or actinomycin D. Upon transfer of these "exhausted" cells to a medium with low levels of amino acids, followed by further incubation, the amount of protease produced in the presence of rifampicin increases linearly and at 75 min reaches a maximum value several times greater than that found in cells used directly from culture. After further incubation the apparent pool of mRNA for protease (as measured by total protease produced) rapidly declined, thereafter showing cyclical oscillations.

In recent years, several examples of extracellular enzyme production showing similar characteristics to the production of extracellular protease by *B. amyloliquefaciens* have been documented. In *Vibrio alginolyticus*, extracellular collagenase production (induced by collagen or its high-molecular-weight fragments) has been shown to occur for 30 to 60 min in the presence of rifampicin at levels which completely inhibit [<sup>3</sup>H]-uracil incorporation into TCA-precip-

itable material (Reid *et al.*, 1980). Labelling studies and controls with chloramphenicol confirmed that collagenase production involves *de novo* synthesis of the enzyme. In a manner similar to that described for protease by Both *et al.* (1972), collagenase production is repressed by Casamino acids. In a similar study, Stinson & Merrick (1974) reported that poly-B-hydroxybutyrate depolymerase secretion by *Pseudomonas lemoignei* continued for 30 min after addition of rifampicin to secreting cultures. These workers suggested that continued secretion may be the result of depolymerase mRNA accumulation within the cells. Extracellular protease secretion by *Pseudomonas maltophilia* is supported for at least 30 min in the absence of mRNA synthesis (Boethling, 1975). Secretion of protease is repressed by pyruvate, L-malate, succinate and most markedly by  $\alpha$ -ketoglutarate; all preferred substrates for growth of the organism. A mechanism similar to catabolite repression was proposed to control exoenzyme secretion. Exoenzyme synthesis was found to be several times more sensitive to inhibitors that affect protein synthesis, than was total protein synthesis. A post-transcriptional control mechanism coupled to  $\alpha$ -ketoglutarate was suggested to account for the finding that rifamycin-insensitive protease secretion could be reversed by either  $\alpha$ -ketoglutarate or chloramphenicol. One further example of prolonged exoenzyme secretion in the presence of inhibitors of transcription is  $\alpha$ -amylase production by the marine bacterium, *Alteromonas rubra* (Gavrilovic *et al.*, 1982). This enzyme is secreted for at least 20 min in the presence of actinomycin D or rifampin at concentrations which completely and rapidly inhibit cellular RNA synthesis,

suggesting the presence of a pool of mRNA specific for the enzyme.

While these examples are similar in several characteristics, there appears to be no known precedent in bacteria for the progressive insensitivity of protease synthesis to transcriptional inhibitors.

The oscillating capacity for rifampicin-insensitive protease production by *B. amyloliquefaciens* can be explained by bursts of protease gene activity alternating with transcriptionally inactive periods. The current working hypothesis put forward by O'Connor *et al.* (1978), is that during gene transcription two forms of protease mRNA are made, one immediately translated, the other forming a reserve pool of non-translatable mRNA which, on cessation of transcription, is converted to the translatable form and supports protease production during the transcriptionally inactive period. Thus it appears as though transcription of the protease gene is switched on when the reserve mRNA pool is low, and off when it is high.

The alternative hypothesis to a reserve pool of mRNA, that of a stable mRNA, while at first sight attractive is in fact exceedingly difficult to correlate with experimental observation. Secretion of protease by cells proceeds at essentially a constant rate irrespective of whether the apparent mRNA pool is very small or very large, implying that mRNA concentration is not rate limiting, i.e., that there is a "reserve" pool. To attempt to explain the observations without this but solely on the basis of a stable mRNA requires extremely complex assumptions. The hypothesis of

Coleman and coworkers (Coleman & Brown, 1975) of a rifampicin-insensitive RNA polymerase is untenable (O'Connor, 1978). The latter paper also discusses the grounds for rejecting other objections by this group of workers.

The deficiency in all of the above work carried out on intact cells is that mRNA pools and sizes must be inferred from the amount of translational product, and at this level of evidence any hypothesis must remain unproven.

Confirmation or rejection of this hypothesis requires examination of protease mRNA production at the molecular level.

It is clear that two levels of control are implicated here: firstly, transcriptional control of protease gene expression; and secondly, if the concept of a reserve mRNA pool for protease is correct, a translational control mechanism of some kind. Speculation as to the possible nature of these controls awaits isolation of the protease gene and direct examination of the apparent protease mRNA pool.

This requires a study by hybridisation analysis of protease mRNA production using the protease gene probe. The main aim of the work in this thesis was to provide a gene probe to allow such a study to be undertaken.

As was mentioned previously, the work presented in this thesis, while related to the question of how extracellular enzymes are produced and transported across the cytoplasmic membranes of bacteria, is directly concerned with the control at the molecular level of a particularly unusual example of this process, that of extracellular protease production by *B. amyloliquefaciens*. This requires isolation of the gene(s)

for these enzymes.

It is of interest therefore to consider examples of the isolation and characterisation of genes for other secretory proteins from *Bacillus*.

The structural gene for the penicillinase of *B. licheniformis* (pen P) has been cloned in both  $\lambda$  phage vectors in *E. coli* (Brammar *et al.*, 1980) and in plasmid vectors in *E. coli* and *B. subtilis* (Gray & Chang, 1981) and its complete DNA sequence determined (Neugebauer *et al.*, 1981). This enzyme exists in both a cell-bound and an extracellular form. The cloned gene was found to be expressed and the precursor protein processed in both *E. coli* and *B. subtilis* (Gray & Chang, 1981). In *B. subtilis* the protein is efficiently secreted whereas in *E. coli* it exists as an outer membrane protein whose properties are indistinguishable from the cell-bound form (Sarvas & Palva, 1983). The signal peptidase cleavage site of the prepencillinase has been determined (Chang *et al.*, 1982) which defines a 26 amino acid NH<sub>2</sub>-terminal signal peptide which is typical of other prokaryote signal peptides in having a hydrophilic NH<sub>2</sub>-terminal region followed by a hydrophobic region (Emr *et al.*, 1980).

The gene for the extracellular amylase of *B. subtilis* has been cloned in  $\lambda$  phage and plasmid pBR322 vectors in *E. coli*, and its DNA sequence determined (Yang *et al.*, 1983). This enzyme is produced as a precursor with a presumed signal peptide of approximately 30 amino acids, which also appears typical of other prokaryote secretory proteins. The cloned gene confers on *E. coli* the ability to degrade starch, indicating its functional expression in this host.

The cloning of a thermostable extracellular neutral protease from *B. stearrowthermophilus* on a vector plasmid in *B. subtilis* is of particular interest to the work presented in this thesis (Fugii *et al.*, 1983). This gene was functionally expressed and the properties of the enzyme from both hosts found to be the same. DNA sequence information, which would allow putative control regions, and the sequence of a signal peptide to be determined, is not yet available for this cloned gene.

Also of particular interest was the cloning of the  $\alpha$ -amylase gene from *B. amyloliquefaciens* and its expression in *B. subtilis* (Palva, 1982). The presence of the cloned gene in *B. subtilis* resulted in about 2500-fold higher production of  $\alpha$ -amylase than wild-type *B. subtilis* and about 5 times more activity than was produced by the donor *B. amyloliquefaciens* strain. The  $\alpha$ -amylase produced from the cloned gene was efficiently secreted by the new host and the enzyme shown to be indistinguishable from that of *B. amyloliquefaciens*. The elevated expression of the cloned gene in the new host can be accounted for partly due to the higher efficiency of the transferred promoter, and partly because of the increased copy number of the gene, due to cloning using the multicopy plasmid pUB110. The promoter and amino-terminal signal peptide region of the cloned  $\alpha$ -amylase gene have been characterised (Palva *et al.*, 1981). This enzyme is produced as a precursor with a signal peptide of 31 amino acids. The upstream control regions of many *B. subtilis* genes have been characterised and consensus sequences determined (Moran *et al.*, 1982). The nucleotide sequence preceding the amino terminus of the  $\alpha$ -amylase gene has properties consistent with the sequences for the RNA-poly-



merase and ribosome-binding sites found in other *B. subtilis* genes.

The gene for the  $\alpha$ -amylase of *B. amyloliquefaciens* has also been transferred to *B. subtilis*, probably as a single copy incorporated into the chromosome, using phage  $\phi$ 3T as vector (Yoneda *et al.*, 1979). A ten-fold increase in  $\alpha$ -amylase production was observed compared with endogenous production (Yang 1980). It was suggested (Palva, 1982) that strain specific control mechanisms for gene expression operate here.

These examples serve to demonstrate that the machinery for expression and transport of secretory proteins is compatible between the *Bacillus* species used here and *E. coli*, although variations in the level of expression occur. In these respects it will be interesting to compare these cloned genes for secretory proteins from *Bacillus* with the protease gene(s) of *B. amyloliquefaciens* when the opportunity arises.

As was mentioned earlier two major species of extra-cellular protease are produced by *B. amyloliquefaciens*, namely alkaline and neutral proteases. In the earlier work of Both *et al.* (1972) and O'Connor (1978) the assay system used measured total protease production. In these studies it was assumed that approximately 90% of this production was neutral protease and that the alkaline protease released by *B. amyloliquefaciens* in culture contributed only a small percentage to the total protease activity. The apparent pool of protease mRNA was accordingly attributed mostly to neutral protease.

Love (1981) in an exhaustive study, rigorously examined the relative contributions of both proteases to total protease

production under various culture conditions, using assay systems capable of distinguishing between the two enzymes. He concluded that the assumption of neutral protease being the predominant species is incorrect and that both the proteases contribute significantly to the apparent pool, their relative contributions varying depending on the culture conditions used. The relative contributions to total protease activity by each protease have been shown to vary between wild type cells grown at 30°C and LF<sup>-</sup> strain <sup>(see page 17)</sup> grown at 20°C, when the apparent pool of protease mRNA is at a maximum. However, under both sets of conditions, the contribution by each protease was never found to be less than 40% of the total protease activity.

Of particular interest with respect to the present work, was the finding that at the time when the apparent pool of protease mRNA is at a maximum, both neutral and alkaline proteases are released when rifampicin is added, the former for the first 30 min and the latter for about 70 min. This prolonged capacity for rifampicin-insensitive alkaline protease synthesis suggests that the apparent mRNA pool for this enzyme may be larger (or more stable) than that of the neutral protease, and points to the control of mRNA production for the alkaline protease [as indicated by Love (1981)] as being the system of choice for initial detailed study at the molecular level.

In using *B. amyloliquefaciens* as a model system for studying protein secretion we are faced with two important questions. Firstly, the fundamental question of how secretory proteins are synthesised and transported across the cytoplasmic membrane of bacteria which was addressed in a recent study in

this laboratory (Love, 1981). The second interesting problem, posed by the findings of our earlier work on the production of proteases by *B. amyloliquefaciens*, is the question of how the production and transport of these secretory proteins occurs and is controlled, in the absence of cellular RNA synthesis. That is, is a reserve pool of mRNA for these proteins indeed maintained within cells, and if so how is this achieved, and how is the synthesis and secretion of these proteins regulated?

The work to be described in this thesis continues the investigation of alkaline protease production by *B. amyloliquefaciens*, the aim being to further our understanding of extra-cellular protease production at the molecular level. The main objective of the work described here was to isolate the gene for the alkaline protease of *B. amyloliquefaciens*, to enable a detailed study by hybridisation analysis of mRNA production under various culture conditions and its possible controls at the transcriptional and translational levels. Progress towards these ends is described in the following chapters.

CHAPTER TWO

MATERIALS AND METHODS

## 2.1 MATERIALS

### BACTERIAL STRAINS

(1) *B. amyloliquefaciens*

An unclassified strain of *B. subtilis* was obtained from the Takamine Laboratories Inc., Clifton, N.J., U.S.A. This organism has since been classified as a distinct species, *B. amyloliquefaciens*, on the basis of DNA base composition, DNA hybridisation studies and transduction experiments by Welker & Campbell, (1967). Two strains of *B. amyloliquefaciens* are used in this laboratory. The wild type (WT) strain produces an extracellular peptide-lipid molecule, "surfactin", that lyses protoplasts (May & Elliott, 1970). A mutant strain, designated LF<sup>-</sup> (Sanders & May, 1975) does not produce this compound. These strains are maintained in this laboratory as spore suspensions prepared as described by Love (1981) and stored at 4°C. Unless otherwise mentioned the LF<sup>-</sup> strain was used in all work presented in this thesis.

(2) *E. coli* MRE 600 used in preparation of cell-free extracts is a Ribonuclease I deficient strain. Stock cultures, prepared by diluting overnight culture 1:1 in 80% (v/v) sterile glycerol are kept at -80°C. Samples were plated for single colonies when required.

(3) *E. coli* MC1061: ara D138, Δ (ara, leu) 7697, Δ lac X 74, gla U<sup>-</sup>, gla K<sup>-</sup>, hsr<sup>-</sup>, hsm<sup>+</sup>, str A (Casadaban & Cohen, 1980) was a gift from R.P. Harvey. This strain was used in transformation experiments and was maintained as described in part (2).

(4) *E. coli* JM101: lac, pro, sup E, thi, F<sup>1</sup> trad D 36, pro AB, lac I<sup>9</sup>, Z Δ M15, was a gift from A.J. Robins. This strain

was used in M 13 cloning experiments and was maintained as described in part (2).

#### GROWTH MEDIA

(1) KYT broth for growth of *E. coli* MRE 600 contains per litre of water; 5.6 gm  $\text{KH}_2\text{PO}_4$ , 28.9 gm  $\text{K}_2\text{HPO}_4$ , 10.0 gm yeast extract, 15 g thiamine, and glucose at 1% (w/v) by adding 50 mls of 20% (w/v) glucose after autoclaving.

#### (2) Growth media for *B. amyloliquefaciens*

(a) Culture medium: The culture medium contained 34 mM  $(\text{NH}_4)_2\text{HPO}_4$ , 1 mM  $\text{MgSO}_4$ , 5 mM KCl, 4.25 mM sodium citrate, 0.125 mM  $\text{CaCl}_2$ , 0.0125 mM  $\text{ZnSO}_4$ , 0.5 M  $\text{FeCl}_3$ , 0.5% (w/v) Bacto casamino acids or acid hydrolysed casein, 0.05% (w/v) Bacto yeast extract (Difco), trace metal solution (0.25 ml/litre) and 1% (w/v) maltose. The medium was adjusted to pH 7.3 with  $\text{H}_3\text{PO}_4$  and sterilised by autoclaving. Maltose was autoclaved separately and was added just before use. The trace metal solution contained 0.5 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.5 mg ammonium molybdate, 5.0 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , and 0.01 mg  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , dissolved in 1 litre of water.

(b) Washed-cell suspension medium: The washed-cell suspension medium was the same as the liquid-growth medium except that  $\text{FeCl}_3$  and yeast extract were omitted thereby limiting cell growth. The medium was prepared with either "high casamino acids" (0.5% w/v) or "low casamino acids" (0.025% w/v).

(c) Protoplast medium: Protoplast medium (PMC) contained 25 mM Tris, 3.8 mM  $(\text{NH}_4)_2\text{HPO}_4$ , 5 mM KCl, 4.25 mM sodium citrate, 0.125 mM  $\text{CaCl}_2$ , 0.0125 mM  $\text{ZnSO}_4$ , 0.025% (w/v)

casamino acids, 0.25 ml/litre of the trace metal solution described for the liquid-growth medium, 1 mM  $MgSO_4$ , 1% (w/v) maltose and 22% (w/v) ribonuclease-free sucrose (Schwartz-Mann, Orangeburg, N.Y.) adjusted to pH 7.3 with HCl.

(3) Luria (L) broth, for growth of *E. coli* MC1061, contains per litre: 10 g Bacto-tryptone (Difco), 5 g yeast extract (Difco), 10 g NaCl and the pH adjusted to 7.2 with NaOH. Where appropriate, the media was supplemented with ampicillin (50 µg/ml) or tetracycline (20 µg/ml).

(4) Growth media for *E. coli* JM101.

(a) Minimal salts media, contains per litre: 10.5 g  $K_2HPO_4$ , 4.5 g  $KH_2PO_4$ , 1.0 g  $(NH_4)_2SO_4$ , 0.5 g  $Na_3$ -citrate, supplemented after autoclaving with 1 ml of 20%  $MgSO_4$ , 0.5 ml of 1% thiamine-HCl and 10 ml of 20% glucose.

(b) 2 X TY broth contains per litre: 16 g Bacto tryptone (Difco), 10 g yeast Extract (Difco), and 5 g NaCl.

(5) Agar plates were prepared by supplementing the above media with 1.5% Bacto agar (Difco). 0.7% agar was used for preparing soft agar overlays. All media was made sterile by autoclaving.

#### ANTIBIOTICS

Rifampicin and ampicillin were from Sigma Chemical Co., Chloramphenicol from Parke Davis & Co., Sydney, Australia, Tetracycline from UPJOHN Pty. Ltd., and sodium azide from BDH Laboratories.

### ISOTOPICALLY LABELLED COMPOUNDS

L-[<sup>35</sup>S]-methionine (1200 Ci/mmol) was from the Radiochemical Centre, Amersham England.  $\alpha$ [<sup>32</sup>P]-dCTP (1500 Ci/mmol),  $\alpha$ [<sup>32</sup>P]-dATP (1500 Ci/mmol),  $\alpha$ [<sup>32</sup>P]-dCTP (1500 Ci/mmol),  $\alpha$ [<sup>32</sup>P]-dGTP (1500 Ci/mmol) and  $\gamma$ [<sup>32</sup>P]-dATP (2000 Ci/mmol) were from Biotechnology Research Enterprises, S.A., Australia.

### ENZYMES

Lysosyme was three-times crystallised from egg-white and was supplied from Sigma Chemical Co. RN'ase A was from Sigma Chemical Co. and was rendered DN'ase free by heating stock solution in water at 80°C for 10 min. Restriction enzymes were from New England Biolabs. Micrococcal nuclease (*Staph. aureus*) and T4 polynucleotide kinase were from Boehringer Mannheim. Reverse transcriptase (RNA-dependent DNA polymerase) was from Molecular Genetics Resources Inc. T4 DNA ligase was from Boehringer Mannheim. Creatine phosphokinase (rabbit muscle) was from Sigma Chemical Co. *E. coli*-DNA-polymerase I, Klenow fragment was from Boehringer Mannheim.

### SYNTHETIC DNA OLIGONUCLEOTIDE PROBE MIXTURES

The two synthetic DNA oligonucleotide probe mixtures, 14-mer 1 and 14-mer 2 (described in Chapter 5, Figure 5.1), were obtained from New England Biolabs and Biotechnology Research Enterprises (B.R.E.S.A.) South Australia, Australia respectively. Custom synthesis for B.R.E.S.A. was performed by Dr. D.C. Skingle.



DETERGENTS

Brij-58 (polyoxyethylene cetylother) and SDS (sodium dodecylsulphate) were obtained from Sigma Chemical Co. Sodium deoxycholate was from BDH Chemicals.

REAGENTS FOR *IN VITRO* SYNTHETIC REACTIONS

All nucleotide triphosphates and deoxynucleotide triphosphates, Creatine phosphate (disodium salt) and DTT (dithiothreitol) were from Sigma Chemical Co. L-<sup>12</sup>C-amino acids were from Mann Research Labs., Inc., N.Y. *E. coli* transfer RNA's (tRNA's) were from the Grand Island Biological Co., N.Y. Hepes (N-2-hydroxy-ethylpiperazine-N'-2-ethan-sulphonic acid) was from Sigma Chemical Co.

OTHER REAGENTS

Crystalline Tris as "Trizma" base, reagent grade and 2-mercaptoethanol were obtained from Sigma Chemical Co. TEMED (N,N,N',N'-tetramethylethylenediamine) was from Eastern Kodak Co. PIPES and calf thymus DNA were from Sigma Chemical Co. and Ammonium persulphate from British Drug Houses (Australia) Pty. Ltd., Victoria. PMSF (phenylmethyl sulphonyl fluoride), acrylamide and bis-acrylamide (N',N'-methylenebisacrylamide) were from Sigma Chemical Co. Other chemicals were routinely obtained from Ajax Chemicals Ltd., and BDH Chemicals Ltd., and were of A.R. grade or of the highest available purity.

### SCINTILLATION FLUID

Scintillation fluid contained 3 g of 2,5-diphenyl oxazole (PPO) and 0.3 g of 1,4-bis-[2-(4 methyl-phenyl oxazolyl)]benzene (POPOP) per litre of toluene. PPO and POPOP were supplied by the Packard Instrument Co., Melbourne, Australia.

### BUFFERS AND MEDIA

Double-distilled water was used throughout except that liquid growth media were prepared with mono-distilled water. The second distillation was from an all-glass apparatus. Solutions were sterilised by autoclaving, except where labile chemicals were involved in which case filtration through Millipore filters was used.

### DIALYSIS TUBING

This was cleaned and made RN'ase-free by boiling in distilled water, in 0.1 M NaHCO<sub>3</sub>, twice more in distilled water, once in 1 mM EDTA, once more in distilled water, and finally washing in distilled water. Tubing was kept in the appropriate buffer if to be used immediately or in 1 mM EDTA if to be stored at 4°C.

### BUFFER SATURATED PHENOL

This was prepared by vigorously mixing equal volumes of redistilled phenol and 0.1 M Tris-Cl, pH 9.5, 5 mM EDTA. The saturated phenol phase was stored at 4°C.

GLASSWARE AND GENERAL EQUIPMENT

All glassware and equipment where necessary was alkali washed (in 1 N KOH, to minimise RNA'se contamination), rinsed well with double-distilled water and where possible sterilised by dry heat or autoclaving.

## 2.2 METHODS

### THE *E. coli* CELL-FREE TRANSLATION SYSTEM

#### Preparation of *E. coli* S30 cell-free extracts

S30 cell-free extracts of *E. coli* MRE600 were prepared by a modification of the procedure by Zubay (1973) as determined by Gunn (1981) and Bawden (1979).

##### (a) Growth of the organism

Ten 2L flasks each containing 500 mls of KYT broth were inoculated with 2 mls of an overnight culture of *E. coli* MRE600 and grown at 30°C with continuous aeration for 3 - 5 hours until A600 nm was in the range 0.5 - 0.6 (early log phase of growth). Cell growth was stopped by addition of the cultures to 2 L of ice and the cells harvested by centrifugation (10,000 g, 10 min). The total cell pellet was stored at -80°C until used.

##### (b) S30 preparation

All operations unless otherwise stated were carried out at 0 - 4°C.

(1) 10 gms of frozen cells (wet weight) were allowed to thaw at 4°C, resuspended in 100 mls of Buffer A and centrifuged. The pellet was resuspended in a further 30 mls of Buffer A and recentrifuged. The cells were then resuspended in 15 mls of Buffer B and lysed by passage once through a pre-chilled alkali-washed French Pressure Cell at 6000 p.s.i. (25  $\mu$ l 1 M DTT was present in the collection tube to maintain reducing conditions). All procedures following cell lysis were carried out in light-protected vessels. The lysate was centrifuged at 30,000 g for 30 minutes and the upper 2/3 - 3/4

volume of the supernatant kept as the S30 fraction.

(2) Pre-incubation of the S30: The S30 fraction was pre-incubated for 80 min at 37°C with gentle agitation at 30 and 60 min after addition of the appropriate amount of pre-incubation cocktail.

(3) Dialysis of the S30: The pre-incubated S30 fraction was then dialysed for 4 h at 4°C against 3 L of Buffer C (three 1 L changes of buffer with 6 mM 2-mercaptoethanol being replaced by 1 mM DTT in the final buffer change). After dialysis, 200 µl aliquots were stored at -80°C ready for use in translation assays. The concentration of protein in these S30 extracts was 30 - 35 mg/ml as determined using the method of Lowry *et al.* (1951). 20 µl of S30 was used per 50 µl assay (after nuclease treatment) as described below.

(4) Nuclease treatment of the S30: Immediately prior to use in the translation assays, each 200 µl aliquot of S30 was treated with micrococcal nuclease as follows. To 200 µl of S30 (thawed on ice) was added 8 µl of 0.1 M CaCl<sub>2</sub> and 32 µl of a 1 mg/ml solution of micrococcal nuclease. This was incubated for 5 min at 37°C after which was added 8 µl of 0.1 M EGTA, and this treated S30 then dispensed immediately into the translation assays.

#### Solutions for *E. coli* S30 extract preparation

(1) Buffer A - 10 mM Tris-acetate, pH 8.2, 14 mM Mg-acetate, 60 mM KCl, 6 mM 2-mercaptoethanol.

(2) Buffer B - As Buffer A except 1 mM DTT replaced the 2-mercaptoethanol.

(3) Buffer C - As Buffer B with 60 mM K-acetate replacing the KCl and DTT or 2-mercaptoethanol being used as indicated

in Methods.

These buffers were prepared on the day to be used by diluting an appropriate 10 x stock solution, adding the appropriate amounts of DTT or 2-mercaptoethanol and adjusting the pH with glacial acetic acid.

(4) Pre-incubation cocktail - contained per 10 ml of S30 extract; 0.460 ml 2 M - Tris-acetate, pH 8.2, 0.010 ml 1 M DTT, 0.030 ml 1 M Mg-acetate, 0.020 ml 5 mM amino acids (stock solution of combined amino acids), 0.075 ml 0.1 M ATP, 0.200 ml 0.5 M creatine phosphate, 0.050 ml of 10 mg/ml creatine phosphokinase.

Solutions (1) - (4) were prepared using double-distilled, deionised sterile water.

Construction and Incubation of *in vitro* reaction mixtures (the *E. coli* cell-free translation system)

The translation assays contained in a final volume of 50  $\mu$ l: 20  $\mu$ l of nuclease treated S30 fraction, 2 mM ATP, 0.25 mM GTP, 10 mM creatine phosphate, 40  $\mu$ g/ml of creatine phosphokinase, 3 mM DTE, 20 mM Hepes (pH 7.6), 60 mM  $\text{NH}_4\text{Cl}$ , 7.5 mM Mg acetate, 200  $\mu$ g/ml of *E. coli* tRNA's, 100  $\mu$ g/ml of folic acid, 31  $\mu$ M unlabelled amino acids (minus methionine), 4  $\mu$ M unlabelled methionine, 10  $\mu$ Ci [ $^{35}\text{S}$ ]-methionine and RNA from *B. amyloliquifaciens* as specified in the figure legends. The assays were incubated at 37°C for 20 min and prepared for electrophoresis as described below.

Preparation of translation products for electrophoresis

Several procedures have been used in the preparation of translation products for electrophoresis including TCA precipitation and acetone precipitation (Bawden, 1979). In

the present work the following procedure was found to give the best results.

Immediately following incubation, the assays (50  $\mu$ l) were made to 100  $\mu$ l in SDS gel loading buffer, (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% Bromophenol blue, 5% 2-mercaptoethanol [added fresh]) by addition of 50  $\mu$ l of 2 X stock buffer, and boiled for 3 min. Unless otherwise mentioned, 20  $\mu$ l of the samples were electrophoresed immediately on SDS-polyacrylamide gels as described below. Following boiling, if required, storage of samples at  $-20^{\circ}\text{C}$  prior to electrophoresis was satisfactory.

#### SDS-Polyacrylamide gel electrophoresis and fluorography

1. 12.5% slab gels were prepared by the method of Laemmli, (1970) which incorporates a stacking gel (3% acrylamide, 0.1% SDS, 0.125 M Tris-HCl, pH 6.8) over a separating gel (12.5% acrylamide, 0.1% SDS, 0.375 M Tris-HCl, pH 8.8). The dimensions of the stacking gel were 1.5 cm x 14 cm x 1.5 mm and those of the separating gel 9 cm x 14 cm x 1.5 mm. A sealing gel (1 cm x 14 cm x 1.5 mm) was always used beneath the separating gel. The pH and acrylamide step-gradients thus formed produced good separation of the proteins into tight, distinct bands.

After loading the samples, electrophoresis was performed at 45 mA until the tracker dye reached the separating gel, (generally 40 min), and then continued at 110 V until the tracker dye entered the sealing gel. During this period (about 3 h), the current dropped steadily to about 10 mA.

After electrophoresis, the gels were kept overnight in 300 mls of a fixative solution of 10% (v/v) acetic acid,

10% (v/v) isopropanol and then processed for fluorography.

2. Fluorography was performed essentially as described by Bonner & Laskey (1974). After fixation overnight the gels were soaked for 60 min in DMSO (two 300 ml volumes, 30 min each) to remove the water, followed by 3 h in 100 mls of a 1.5% (w/v) PPO, 25% (w/v) naphthalene solution in DMSO. The gels were then soaked for 90 min in three changes of water to precipitate the PPO/naphthalene in the gel, after which they were dried down onto 3 mm Whatman paper using a slab gel dryer (Hoefer Scientific Instruments). Unless otherwise mentioned in the text, the gels were autoradiographed at  $-80^{\circ}\text{C}$  overnight.

3. To estimate [ $^{35}\text{S}$ ]-methionine incorporation into TCA precipitable material in the translation assays, 5  $\mu\text{l}$  of each gel sample was pipetted into 1 ml of cold 10% (w/v) TCA containing 0.5% (w/v) methionine and 5  $\mu\text{l}$  of 10% (w/v) BSA as carrier. This was allowed to stand on ice for 15 min and then heated at  $90 - 95^{\circ}\text{C}$  for 15 min to hydrolyse aminoacyl-tRNA's. After a further 30 min on ice the TCA precipitates were collected onto GF/A filters using the Millipore suction apparatus, washed 5 times with 5 mls of 10% (w/v) TCA, 0.5% (w/v) methionine, the filters dried under a heat lamp and counted by liquid scintillation.

#### Preparation of [ $^{35}\text{S}$ ]-methionine labelled culture supernatant proteins of *B. amyloliquifaciens*

The [ $^{35}\text{S}$ ]-methionine labelled culture supernatant protein markers of *B. amyloliquifaciens* used in Chapters 3 and 4 were prepared as described below.

20 mls of *B. amyloliquifaciens* LF<sup>-</sup>culture (OD600 nm



≈ 3.6) was centrifuged (4,000 r.p.m., 3.5 min, MSE bench centrifuge) and the cell pellet washed twice by resuspension in low amino acids medium. 10 mls of the final resuspension was placed in a 100 ml conical flask and 60  $\mu\text{Ci}$  [ $^{35}\text{S}$ ]-methionine (1200 Ci/mmol) added and the suspension incubated at 30°C for 45 min with vigorous shaking (360 o.p.m.). The cells were then pelleted and 1 g of TCA dissolved in the culture supernatant which was kept on ice for a further 45 min to precipitate the protein. The precipitate was pelleted by centrifugation (15 min, 4,500 r.p.m., MSE bench centrifuge) and washed twice to remove unincorporated label by resuspension in 2 mls of 10% (w/v) TCA, 0.05% (w/v) methionine. The TCA was removed by washing in 90% (v/v) ethanol (3 x) and then ether (2 x). The precipitate was air dried and finally dissolved in 400  $\mu\text{l}$  of SDS gel loading buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.001% Bromophenol blue, 5% 2-mercaptoethanol [added fresh]) and boiled for 5 min. 20  $\mu\text{l}$  of this culture supernatant protein preparation, when electrophoresed on an SDS-polyacrylamide gel, gave readily visible marker bands after overnight autoradiography.

#### PREPARATION OF RNA FROM *BACILLUS AMYLOLIQUIFACIENS*

RNA was routinely prepared from 120 mls of culture using an extraction procedure employing guanidine-hydrochloride as detailed below.

The cell pellet from 120 mls of culture was resuspended in 10 mls of solution G1 at 4°C (6 M guanidine-HCl, 0.2 M sodium-acetate, pH 5.2, 5 mM 2-mercaptoethanol) and the suspension passed through a chilled French Pressure Cell at 12,000 p.s.i. The lysate was homogenized with an equal

volume of 95% ethanol (redistilled) using a Dounce glass homogenizer, and stood at  $-20^{\circ}\text{C}$  for one hour. The mixture was then centrifuged for 20 minutes at 14 K,  $4^{\circ}\text{C}$  (Sorval SS-34 rotor) and the pellet homogenized in 6 mls solution G2 at  $4^{\circ}\text{C}$  (6 M guanidine-HCl, 0.2 M sodium-acetate, pH 5.2, 10 mM EDTA, 5 mM 2-mercaptoethanol). After homogenizing again with an equal volume of 95% ethanol the mixture was stood at  $-20^{\circ}\text{C}$  for a further one hour, and then recentrifuged. The pellet was homogenized in 3 mls Urea solution (7 M urea [recrystallised], 0.2 M Tris-HCl, pH 8.5, 0.1 mM EDTA, 0.1% SDS) at room temperature and extracted twice with equal volumes of buffer saturated phenol and chloroform, making the aqueous phase to 1% SDS (w/v) for the second extraction. The aqueous phase was finally extracted once with an equal volume of chloroform, made to 0.1 M K-acetate, pH 5.0 and the RNA precipitated at  $-20^{\circ}\text{C}$  overnight in the presence of two volumes of ethanol. The precipitate was pelleted by centrifugation at 10 K for 20 min, resuspended in 5 mls 2 M LiCl and recentrifuged. The pellet was again resuspended, in 5 mls 70% ethanol-30% 0.1 M K-acetate, pH 5.0, centrifuged again, dried under a light stream of nitrogen gas and the RNA finally dissolved in sterile glass-distilled water and stored in aliquots at  $-80^{\circ}\text{C}$ .

#### Optimisation of the apparent protease mRNA pool of *B.*

##### *amyloliquifaciens*

Preparation of RNA from *B. amyloliquifaciens* cells with a minimal or high apparent protease mRNA pool required optimisation of cell cultures for these two sets of conditions and was carried out as described by Love (1981) as detailed

below.

(a) Growth of the cells

Culture medium was inoculated with a platinum loop from a suspension of spores of LF<sup>-</sup> strain *B. amyloliquifaciens* and incubated at 20°C under sterile conditions in an orbital shaker (Model 461, Paton Industries, S.A.) at 300 - 350 cycles per min. Cultures were harvested at  $OD_{600\text{ nm}}^{1\text{ Cm}} = 3.6$ , which corresponds to late-logarithmic phase (approximately 72 h after inoculation).

(b) Incubation in high amino acids suspending medium

The cells from 240 mls of late-log phase culture were harvested, washed by resuspension and centrifugation (twice) at 30°C with high amino acids suspending medium. After final resuspension in fresh medium the cells were shaken in 40 ml lots in 250 ml conical flasks at 30°C in an orbital shaking water bath (Model OW1412, Paton Industries, S.A.) at 380 cycles per minute for 75 minutes.

At this point cells from 120 mls of culture were harvested and the RNA isolated. Cells at this stage have a minimal apparent protease mRNA pool and the RNA isolated is designated 75 Haa RNA (Refer to Chapter 3).

(c) Incubation in low amino acids suspending medium

Cells from the remaining 120 mls of culture were harvested, washed, and incubated as in (b) in low amino acids suspending medium for 60 min.

Cells at this stage have an apparent protease mRNA pool approaching its maximal level, and the RNA isolated is designated 60 Laa RNA.

### Gel Electrophoresis of RNA

Electrophoresis of RNA for analytical or preparative purposes, or for transfer, was carried out using vertical slab gels (14 cm x 14 cm x 0.3 cm) containing 1% (w/v) agarose and 5 mM methylmercury hydroxide. Electrophoresis buffer consisted of 50 mM boric acid, 5 mM Na-tetraborate, 10 mM Na<sub>2</sub>SO<sub>4</sub>, pH 8.2, and the RNA samples were loaded in a solution composed of the electrophoresis buffer, 10% (w/v) glycerol, 5 mM methylmercury hydroxide, 0.001% Bromophenol blue and 0.001% xylene cyanol. Electrophoresis was carried out at 50 mA for about 3 h and the RNA visualised by staining with 0.02% (w/v) ethidium bromide and examination under UV light.

Methylmercury hydroxide is toxic and therefore all operations were carried out in a fume hood, using gloves, and the methylmercury neutralised as soon as practicable by immersion of all equipment in a solution of 1 mM 2-mercapto-ethanol. Equipment was treated to minimize ribonuclease contamination as described in Materials.

### GRADIENT ANALYSIS OF RNA

#### (a) Sucrose gradient analysis of RNA

Linear density gradients of 10 - 40% (w/v) sucrose in TEN buffer (20 mM Tris-HCl, pH 8.5, 100 mM NaCl and 1 mM EDTA) were prepared using a gradient former. The sucrose solutions were prepared using stocks of 50% (w/v) ribonuclease-free sucrose, 5 times concentrated TEN buffer and sterile glass-distilled water. The gradients were centrifuged at 4°C in a Beckman L265B ultracentrifuge (SW41 rotor) for the

times and speeds indicated in the figure legends. A254 profiles were obtained using an Isco density gradient flow cell, and fractions were collected manually. The RNA samples were in TEN buffer. To check the intactness of the RNA, samples were dissolved in TEN buffer containing 0.2% (w/v) SDS and heated at 60°C for 2 min before loading on to gradients.

(b) Sucrose/formamide gradient analysis of RNA

Linear density gradients of 5 - 25% (w/v) sucrose in 70% (v/v) formamide, 3 mM Tris-HCl, pH 7.6, 3 mM EDTA, were prepared using a gradient former. The gradients were centrifuged on a Beckman L8-70 ultracentrifuge (SW41 rotor) at 20°C for the times and speeds indicated in the figure legends. A280 profiles were obtained using an Isco density gradient flow cell, and fractions were collected manually. The RNA samples were incubated at room temperature for 5 min in the presence of methylmercury hydroxide (5 mM final concentration) just prior to loading on the gradients.

(c) Recovery of the RNA from gradient fractions

To recover the RNA, gradient fractions were made to 0.1 M K-acetate, pH 5.0, two volumes of ethanol added and the RNA precipitated overnight at -20°C. The RNA was pelleted (by centrifugation at 10 K, 20 min, Sorvall SS34 rotor), dissolved in 4 mls of sterile glass distilled water and reprecipitated. The final RNA pellet was rinsed in 70% (v/v) ethanol, dried under vacuum, dissolved in a small volume of water and stored in aliquots at -80°C.

Transfer of RNA to nitrocellulose (Northern analysis)

RNA fractionated in 1% agarose/methylmercury gels was transferred to nitrocellulose using a procedure based on that of Thomas (1980).

After running the gels, to reduce the salt concentration in the gel prior to transfer, and to neutralise the methylmercury, the gel was soaked twice for 40 min each in 200 mls of 10 mM Na PO<sub>4</sub>, pH 7.0, 5 mM 2-mercaptoethanol at room temperature, twice for 10 min in 200 mls of 10 mM NaPO<sub>4</sub>, pH 7.0, 7 mM iodoacetic acid, and finally twice for 5 min in 200 mls of 10 mM Na PO<sub>4</sub>, pH 7.0. The gel was then placed on 2 sheets of Whatman 3 mm paper saturated with 20 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M Na citrate), and gladwrap placed around the edges of the gel. Nitrocellulose (Gene Screen hybridisation transfer membrane, cut to the appropriate size and preboiled) was placed on top of the gel and covered with 2 sheets of dry Whatman 3 mm paper and paper-towels to a thickness of about 3 cm, and weighted down. Transfer was allowed to proceed overnight at room temperature, after which the nitrocellulose was air dried and baked 2 h at 80°C under vacuum prior to use in hybridisation experiments.

#### HYBRID-SELECTION TRANSLATION PROCEDURE

Clones were characterised by hybrid-selection translation (Chapter 4) using a procedure based on those of Ricciardi *et al.* (1979) and Bozzoni *et al.* (1981) as described below.

##### (a) Preparation of nitrocellulose filters

Millipore nitrocellulose filters (type GS, 0.22 µm) were boiled three times in sterile glass-distilled water. 40 µg of plasmid DNA of each clone was boiled 5 min in 0.5 ml of 0.15 N NaOH, neutralised with 50 µl of 1.5 N HCl and diluted to 10 ml in 2 M NaCl. The DNA was loaded onto filters rinsed in 2 M NaCl using a Millipore suction apparatus (gentle

suction), and the filters washed twice more with 10 ml of 2 M NaCl. The filters were air dried and baked at 80°C under vacuum for 2 h.

(b) Hybridisation with *B. amyloliquifaciens* RNA and washing of filters

The filters were hybridised at 50°C for 2 h in Eppendorf tubes, with 200 µl of a hybridisation solution composed of 10 mM PIPES NaOH, pH 6.4, 0.4 M NaCl, 0.2% SDS, containing 100 µg of 75 Haa RNA of *B. amyloliquifaciens*. After hybridisation, the hybridisation solution was aspirated and the filters washed 10 times with 0.5 ml of 1 X SSC, 0.2% SDS at 60°C and then 2 times with 1 ml 0.2 X SSC, 0.2% SDS at 60°C (no SDS in second wash). The filters were then incubated 5' at 50°C in 1 ml of 20 mM Tris-acetate, pH 7.6, 5 mM NaCl, 2 mM EDTA as a final wash.

(c) Elution and recovery of bound RNA

RNA remaining bound to the filters was eluted by adding 0.5 ml of boiling sterile glass-distilled water and maintaining the heat for 2 min. The water was taken into a fresh tube, freeze dried to reduce the volume to about 50 µl and the RNA precipitated overnight at -80°C after addition of 3 µl of 2 M K-acetate, pH 5.0, and 150 µl ethanol. The RNA was pelleted by centrifugation (30 min, Eppendorf microfuge, 4°C), rinsed with 70% ethanol, dried under vacuum and finally dissolved in 10 µl of water for immediate translation.

(d) Translation of the RNA

The eluted RNA was translated as described previously in the *E. coli* cell-free system and the translation products analysed by SDS-polyacrylamide gel electrophoresis and

fluorography, as indicated in the figure legends.

#### PREPARATION OF ANTI-ALKALINE PROTEASE ANTISERA

Antisera to pure alkaline protease of *B. amyloliquefaciens* (O'Connor, personal communication) was raised in rabbits by injection of 1 mg of antigen mixed with an equal volume of Freund's complete adjuvant, into 4 subcutaneous sites. Booster injections of 0.5 mg antigen were given 7 days and 21 days after the initial injection. 4 days later, blood was collected by bleeding at the ear. After clotting at 37°C for 1 h, the clot was allowed to contract for 12 h at 4°C, and the clear serum carefully collected and made to 0.1% sodium azide. The serum was tested against the pure antigen using standard Ouchterlony immunodiffusion techniques and used in immunoprecipitation experiments without further purification.

#### Immunoprecipitation trials

Immunoprecipitation trials using the alkaline protease antisera, were carried out using a procedure based on that of Matsuura *et al.* (1981). [<sup>35</sup>S]-methionine labelled samples were made to 1 ml in immunoprecipitation buffer (50 mM Tris-Cl, pH 8.0, 0.15 M NaCl, 1% triton X-100, 1% deoxycholate, 1 mM EDTA). Antisera (10 µl or 20 µl) was added and the samples incubated 15 min at room temperature, then overnight at 4°C. Immunoprecipitates were collected by adsorption to *S. aureus* cells based on the method of Kessler (1975). *S. aureus* cells (Cowan 1 strain) were purchased from C.S.L. Laboratories, Melbourne, Australia, and washed thoroughly in immunoprecipitation buffer prior to use. The washed cells were finally resuspended as a 10% suspension



and 100  $\mu$ l added to the immunoprecipitation mixtures. Incubation was allowed to proceed for 1 h at 4°C. The *S. aureus* cells were pelleted by centrifugation (10,000 xg for 2 min) and washed twice in immunoprecipitation buffer. The pelleted cells were then resuspended in 50  $\mu$ l of SDS-polyacrylamide gel loading buffer and heated at 100°C for 2 min. After centrifugation, the supernatant was analysed by SDS-polyacrylamide gel electrophoresis as described previously.

#### PREPARATION OF *B. AMYLOLIQUIFACIENS* DNA

Chromosomal DNA of *B. amyloliquifaciens* was prepared using a modification of the procedure of Marmur (1961) as described below.

240 mls of *B. amyloliquifaciens* LF<sup>-</sup> culture (OD600  $\approx$  3.6) were converted to protoplasts by incubation in the presence of lysosyme (180  $\mu$ g/ml final concentration) for 25 min in protoplast medium, as described by Love (1981). The protoplasts were collected by centrifugation (8,000 xg, 5 min, MSE centrifuge) and resuspended in 10 mls of a solution of 10 mM Tris-Cl, pH 8.0, 10 mM NaCl, 10 mM EDTA, 0.5% (w/v) SDS containing protease K at a final concentration of 50  $\mu$ g/ml. This was incubated with gentle shaking at 37°C for 2 h, and then extracted three times with buffer saturated phenol. DNA was precipitated from the aqueous phase overnight at -20°C after addition of 0.1 ml of 3 M Na-acetate, pH 5.5 and two volumes of absolute ethanol. The DNA was pelleted by centrifugation (14 K, 30 min, Sorval SS34 rotor) and dissolved in 1 X SSC. To digest RNA, DNA'se free RNA'se was added to a final concentration of 20  $\mu$ g/ml and the solution incubated at 37°C for 2 h. The phenol extractions were

repeated and phenol removed by a final extraction with chloroform prior to ethanol precipitation again. The DNA was stored at  $-20^{\circ}\text{C}$  as an ethanol precipitate in aliquots of 500  $\mu\text{g}$ . Prior to use, the DNA was pelleted, washed with 70% (v/v) ethanol and then absolute ethanol, dried under vacuum and dissolved in the desired volume of 0.1 mM EDTA. Material in use was kept at  $4^{\circ}\text{C}$ .

### CONSTRUCTION OF COLONY LIBRARIES OF *B. AMYLOLIQUIFACIENS*

#### DNA IN *E. coli*

##### (1) Preparation of pBR322 plasmid vectors

In the work presented in this thesis two plasmid vectors were used; *Bam*HI digested pBR322 and *Hind*III digested pBR322. These were prepared as follows; 5  $\mu\text{g}$  of plasmid DNA was digested in a 20  $\mu\text{l}$  reaction containing a four fold excess of the appropriate enzyme for 3 h at  $37^{\circ}\text{C}$ . Digestion was checked by running 0.5  $\mu\text{l}$  of the reaction mix on an agarose minigel. To prevent self-ligation of the vector, the 5' terminal phosphate groups of linear molecules were removed by a further incubation for 1 h at  $37^{\circ}\text{C}$  after addition of 166  $\mu\text{l}$  of water, 2  $\mu\text{l}$  of 1 M Tris-Cl, pH 9.0 and 10  $\mu\text{l}$  (5 units) of calf intestinal phosphatase. Following this incubation the reaction was phenol extracted and ethanol precipitated. The DNA was finally electrophoresed on a preparative low melting temperature agarose minigel. A gel slice containing linear DNA was excised from the gel and the DNA extracted from the agarose as described later in this Chapter. The vector DNA was finally dissolved in 0.1 mM EDTA at a concentration of 50 ng/ $\mu\text{l}$  for use in ligation and transformation experiments. For short term use aliquots of the

vector preparations were stored at 4°C and at -20°C for long term storage.

(2) Ligation reactions

*B. amyloliquifaciens* DNA prepared as described in the text was ligated into the restriction site of the appropriate pBR322 plasmid vector in 10 µl reactions containing 50 ng of vector DNA, an equimolar amount of *B. amyloliquifaciens* DNA, 50 mM Tris-Cl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM rATP and 1 unit of T<sub>4</sub> DNA ligase. The reactions were incubated at 4°C for from 12 to 24 h. A control ligation with no added *B. amyloliquifaciens* DNA was included to determine background levels.

(3) Transformation of *E. coli*

*E. coli* strain MC1061 (see Materials) was grown overnight at 37°C in Luria broth and then diluted 1/50 into fresh Luria broth and grown to an A<sub>600</sub> of 0.6 - 0.8. The cells were chilled on ice for 30 min, pelleted by centrifugation and resuspended in 1/2 volume of ice-cold 0.1 M MgCl<sub>2</sub>. The cells were pelleted immediately and resuspended in 1/20th of the original volume of ice-cold 0.1 M CaCl<sub>2</sub>. The cells were kept on ice for at least one hour. 0.2 ml of these competent cells was added to 0.1 ml of the ligated DNA (aliquots of 2 - 10 µl) in 0.1 M Tris-Cl, pH 7.4 and stood on ice for 30 min with occasional stirring. The cells were heat-shocked at 42°C for 2 min, kept on ice for a further 30 min and then warmed slowly to room temperature. 0.5 ml of Luria broth was added to the transformed cells and incubated at 37°C for 20 min. The transformed cells were mixed with 3 mls of 0.7% L-agar and plated on 1.5% L-agar plates containing the appropriate antibiotic (50 µg/ml of ampicillin or 20 µg/ml

of tetracycline). These were incubated overnight at 37°C.

## DETECTION AND EXAMINATION OF RECOMBINANT TRANSFORMANT

### COLONIES

#### (1) Ordered colony screening

Nitrocellulose filters for screening colonies in an ordered array (Chapter 4) were prepared essentially as described by Grunstein and Hogness (1975). Colonies from a transformation were transferred by toothpick to a master plate and to a sheet of nitrocellulose that had been boiled three times in distilled water and laid onto an L-agar plate containing the appropriate antibiotic. The colonies were grown overnight on the nitrocellulose at 37°C, and the colonies lysed by transferring the nitrocellulose sequentially onto 3 mm paper saturated with 0.5 N NaOH for 7 min, 1 M Tris-Cl, pH 7.4 for 2 min, 1 M Tris-Cl, pH 7.4 for 2 min and and 1.5 M NaCl, 0.5 M Tris-Cl, pH 7.4 for 4 min. The nitrocellulose filter was washed in 95% ethanol and then baked at 80°C, under vacuum, for 2 h. Hybridisation and washing conditions were as described to follow.

#### (2) High density colony screening

Nitrocellulose filters for screening colonies at high density (Chapter 5) were prepared essentially as described by Hanahan and Meselson (1980). Colonies from a transformation were transferred to a nitrocellulose filter by gently overlaying the nitrocellulose onto the original plate. Careful removal of the nitrocellulose resulted in transfer of cells to the nitrocellulose while enabling regrowth on the original plate. The colonies were grown for several hours on the

nitrocellulose after placing the filter on a fresh L-agar plate containing the appropriate antibiotic, until small colonies were apparent. The nitrocellulose was then placed on a fresh L-agar plate containing 200 µg/ml chloramphenicol and incubated overnight at 37°C to amplify the plasmid DNA of the recombinants. The colonies were lysed and prepared for hybridisation as described in Section (a) above.

### (3) Preparation of glycerol stocks

Recombinant pBR322 clones in *E. coli* strain MC1061 were grown overnight at 37°C in L broth containing the appropriate antibiotic at 50 µg/ml final concentration. Single 5 ml cultures or 0.1 ml cultures in 96 well trays were used. After growth, cultures were mixed 1:1 with 80% (v/v) glycerol and stored at -20°C or at -80°C for long term storage.

## PREPARATION OF RECOMBINANT PLASMID DNA

### (1) Mini-preparations of plasmid DNA

Isolation of plasmid DNA from small cultures was carried out as follows. 1 ml cultures of each recombinant were grown overnight in L-broth containing the appropriate antibiotic (50 µg/ml ampicillin or 20 µg/ml tetracycline). The cells were pelleted by centrifugation for 2 min in an Eppendorf centrifuge, all liquid media removed, and the cells resuspended in 50 µl T.E.S. solution (25 mM Tris-Cl, pH 8.0, 10 mM EDTA, 15% sucrose) containing lysosyme (freshly added) to 2 mg/ml. The suspension was incubated on ice for 5 min and then 100 µl 0.2 N NaOH, 1% SDS added, mixed gently and kept 10 min on ice. 75 µl of 3 M Na acetate, pH 4.6 was added, mixed gently and incubation continued on ice for 10

min. The solution was centrifuged 5 min and the supernatant removed to a fresh Eppendorf tube, 1  $\mu$ l of DNA'se-free RNA'se A added (10 mg/ml stock) and incubated at 37°C for 30 min. After extraction twice with buffer-saturated phenol: chloroform (1:1) and finally chloroform, the DNA was ethanol precipitated at -80°C for 30 min in 3 volumes of ethanol. The DNA was pelleted by centrifugation for 10 min, washed in 70% ethanol, 95% ethanol, dried under vacuum and finally dissolved in 10  $\mu$ l of water. 2  $\mu$ l of this preparation is sufficient for DNA restriction analysis.

(2) Large scale preparation of recombinant plasmid DNA

A single colony of *E. coli* containing the recombinant plasmid was used to inoculate 5 mls of L-broth containing the appropriate antibiotic (50  $\mu$ g/ml ampicillin or 20  $\mu$ g/ml tetracycline) and grown overnight at 37°C with vigorous shaking to provide aeration. The overnight culture was diluted 100-fold in fresh media and grown with aeration at 37°C to an A600 of 0.6 - 0.8, at which time chloramphenicol was added to a final concentration of 200  $\mu$ g/ml and incubation continued with aeration at 37°C overnight.

The cells were pelleted by centrifugation (10,000 g for 10 min) and resuspended in 4 mls TS solution (50 mM Tris-Cl, pH 8.0, 25% sucrose). 1 ml of 5 mg/ml lysosyme in TS was added, mixed in gently and the solution kept on ice for 5 min. 2 mls of 0.25 M EDTA, pH 8.0 was mixed in gently and incubation continued on ice for 5 min. 8 mls of detergent solution (50 mM Tris-Cl, pH 8.0, 1% BRIJ58, 0.4% Na deoxycholate, 25 mM EDTA) was then added, mixed in gently by inversion of the tube and kept on ice a further 10 min. The solution was then centrifuged (18 K, 30 min, Sorval SS34 rotor) and the clear

supernatant removed into a fresh container.

Caesium chloride (0.95 g/ml of supernatant) was dissolved in the supernatant and 20  $\mu$ l of 10 mg/ml ethidium bromide per ml of supernatant added. This solution was centrifuged for 44 h, 44 K, 20°C (Ti 50 rotor, Beckman L8-70 ultracentrifuge). The tubes were punctured at the bottom and the lower band of supercoiled plasmid DNA, (visualised by the ethidium staining) was collected. A second centrifugation was carried out if required to remove nicked DNA. To remove the ethidium bromide the solution containing the DNA was extracted with equal volumes of isopropanol saturated with 5 M NaCl, 10 mM Tris-Cl, pH 8.5, 1 mM EDTA, until all pink coloration was removed. The DNA was finally precipitated in 3 volumes of ethanol twice, washed in 70% ethanol, 95% ethanol, dried under vacuum and dissolved in a small volume of 0.1 mM EDTA for storage at 4°C.

#### RESTRICTION ANALYSIS OF DNA.

All restriction endonuclease digestion of DNA was carried out using the conditions for the appropriate enzymes detailed in the New England Biolabs catalogue. For analytical purposes 1  $\mu$ g of plasmid DNA or 5  $\mu$ g of genomic DNA was digested in a 10  $\mu$ l reaction. After incubation, the reaction was made up in gel loading buffer (2 M Urea, 20% sucrose, 5 mM EDTA, 0.005% Bromophenol blue) by addition of 5  $\mu$ l of a 3 X stock loading solution, in preparation for electrophoresis. Preparative restriction digests were carried out in large scale reactions as specified in the appropriate figure legends. For digests involving more than one restriction enzyme, the enzymes were

added together if digestion buffers were the same, or if different, the reactions were phenol extracted and the DNA ethanol precipitated prior to incubation with the next enzyme.

#### Agarose Gel electrophoresis of DNA.

Electrophoresis of DNA for analytical purposes or for transfer to nitrocellulose was carried out using vertical slab gels (14 cm x 14 cm x 0.3 cm) or flat bed minigels (poured on 7.5 cm x 5.0 cm glass microscope slides) containing 1% (w/v) agarose. Electrophoresis buffer consisted of 40 mM Tris-acetate, pH 8.2, 20 mM Na-acetate, 1 mM EDTA and electrophoresis was carried out at 60 mA (about 3 h for the slab gels and 40 min for the minigels) DNA was visualised by staining with 0.02% (w/v) ethidium bromide solution for 15 min and examination under UV light.

#### Transfer of DNA from agarose gels to nitrocellulose (Southern analysis)

Restricted DNA fractionated on 1% agarose gels was transferred to nitrocellulose using the method of Southern (1975), as modified by Wahl *et al.* (1979) using 1 M  $\text{NH}_4^-$  acetate, 0.02 M NaOH for neutralisation and transfer as recommended by Smith & Summers (1980), as detailed below.

Gels were soaked in two changes of 250 mls of 0.25 M HCl for 10 min each. The acid was decanted and the gels rinsed briefly in distilled water. This process was repeated using 0.5 M NaOH, 1 M NaCl, again rinsing in distilled water. Gels were neutralised in two changes of 250 mls 1 M  $\text{NH}_4^-$  acetate, 0.02 M NaOH for 15 min each. The gels were then placed on top of two sheets of Whatman 3 MM paper saturated with 1 M



NH<sub>4</sub>-acetate, 0.02 M NaOH, and Glad-wrap placed around the gel. Nitrocellulose paper (cut to the appropriate size and presoaked for 15 min in distilled water) was placed carefully on top of the gel ensuring that no air bubbles are trapped. Two sheets of dry 3 MM paper were placed on top of the nitrocellulose followed by two inches of Kleenex paper towel and weight. Transfer of DNA to the nitrocellulose paper was allowed to proceed overnight. The nitrocellulose paper was finally baked 2 h at 80°C under vacuum to bind the DNA prior to hybridisation.

#### Preparation of *Hind*III digested $\lambda$ DNA markers

*Hind*III digested  $\lambda$  DNA was used to provide markers of known size in agarose gel electrophoresis. 20  $\mu$ g of  $\lambda$  DNA was digested in a 40  $\mu$ l reaction with a four-fold excess of *Hind*III for 3 h at 37°C. Digestion was checked by running a 0.5  $\mu$ l aliquot of the reaction mix on an agarose minigel. After incubation the reaction was heated at 65°C for 5 min and made up to 400  $\mu$ l in gel loading buffer (2 M Urea, 20% sucrose, 5 mM EDTA, 0.005% Bromophenol blue) by addition of 134  $\mu$ l of a 3 X stock loading solution and 226  $\mu$ l of water. This preparation of markers was stored at -20°C when not in use. 20  $\mu$ l contains 1  $\mu$ g of *Hind*III digested  $\lambda$  DNA for use as standard size markers.

#### RECOVERY OF RNA AND DNA FROM LOW MELTING TEMPERATURE AGAROSE GEL SLICES

The slices from 1% low melting temperature agarose gels (0.5 g agarose or less) were placed in Eppendorf tubes (1.5 ml), 200  $\mu$ l NET buffer added (20 mM Tris-HCl, pH 7.4, 0.2 M

NaCl, 2 mM EDTA) and the samples heated at 65°C for 5 min to melt the agarose. 0.5 ml of buffer saturated phenol (see Materials) was added, the samples vortexed vigorously and centrifuged 5 min in the Eppendorf microfuge. The aqueous phase was transferred to a fresh tube and re-extracted twice more with buffer saturated phenol, and finally with chloroform to remove traces of phenol. The RNA or DNA was recovered from the final aqueous phase by ethanol precipitation. For RNA, 2 M K-acetate, pH 5.0, was added to a final concentration of 0.1 M, two volumes of ethanol added, and the RNA precipitated overnight at -20°C. For DNA, 3 M Na-acetate, pH 5.5 was added to a final concentration of 0.3 M, three volumes of ethanol added and the DNA precipitated at -80°C for 30 min or overnight. The RNA or DNA was pelleted by centrifugation (10 min, Eppendorf microfuge), rinsed with 70% (v/v) ethanol, dried under vacuum, and dissolved in a small volume of sterile glass distilled water (for RNA) or in 0.1 mM EDTA for DNA.

#### DOT BLOT ANALYSIS OF RNA OR SINGLE STRANDED (ss) DNA

For dot blot analysis of RNA or ss DNA, nitrocellulose filters were prepared for hybridisation as described below. Hybridisations with the appropriate [<sup>32</sup>P]-labelled probes were carried out as to be described and as detailed in the figure legends.

Samples were made to 10 µl in 1 M NH<sub>4</sub> acetate. The sample nitrocellulose paper was presoaked in water and placed on top of a similarly treated piece of nitrocellulose to provide a flat surface for sample loading. The nitrocellulose sheets were placed on an absorptive pad composed of 2 sheets of Whatman 3 MM paper under which was Kleenex paper towelling to a thickness of about 1 inch. The sample nitrocellulose was

rinsed with 1 M  $\text{NH}_4$ -acetate. The 10  $\mu\text{l}$  samples were hand loaded in a small area using a fine capillary, allowing the liquid to drain through slowly. After loading all samples, the filter was rinsed again in 1 M  $\text{NH}_4$ -acetate, air dried, and baked for 2 h at 80°C under vacuum prior to use in hybridisation experiments. Where required double stranded DNA (ds DNA) was rendered single stranded by heating at 80°C for 10 min in the presence of 0.4 N NaOH.

#### PREPARATION OF [ $^{32}\text{P}$ ]-LABELLED DNA PROBES

##### (a) Random prime cDNA synthesis

Random prime [ $^{32}\text{P}$ ]-cDNA was made using *B. amyloliquifaciens* partially purified mRNA fraction 4 (prepared as discussed in Chapter 3) in the following reaction mix. The 50  $\mu\text{l}$  reaction contained 5  $\mu\text{l}$  of the mRNA preparation, 20  $\mu\text{l}$  of Taylor primer, 50 mM KCl, 3 mM  $\text{MgCl}_2$ , 8 mM DTT, 45 mM Tris-Cl, pH 8.3, 100  $\mu\text{g/ml}$  actinomycin D, 0.8 mM dATP, 0.8 mM dTTP, 100  $\mu\text{Ci}$  [ $^{32}\text{P}$ ]-dGTP (1000 Ci/mmol), 100  $\mu\text{Ci}$  [ $^{32}\text{P}$ ]-dCTP (1000 Ci/mmol) and 1 unit of reverse transcriptase, and was incubated at 37°C for 2 h. 2.5  $\mu\text{l}$  of 10 N NaOH was then added and incubation continued at 42°C for 1 h, to hydrolyse the RNA. The reaction was neutralised by addition of 10  $\mu\text{l}$  1 M Tris-Cl, pH 7.6, and 15  $\mu\text{l}$  1 N HCl, and 10  $\mu\text{l}$  of 0.1 M EDTA added. The cDNA was separated from free label by passage down a G-50 column. Fractions containing [ $^{32}\text{P}$ ]-cDNA were pooled and freeze dried prior to use in hybridisation experiments.

##### (b) Synthesis of rRNA probes

Random prime [ $^{32}\text{P}$ ]-cDNA was made as for (a) in reactions using 1  $\mu\text{g}$  of 16 S rRNA or 23 S rRNA of *B. amyloliquifaciens*.

These RNAs were isolated by electrophoresis of 20  $\mu$ g of total RNA on a 1% low melting temperature agarose/methylmercury gel and isolation of the rRNA bands from the agarose as described elsewhere in this chapter.

(c) Synthesis of extended cDNA probes using synthetic oligonucleotide primers

The synthetic DNA 14-mers described in Figure 5.1 were used as primers in the synthesis of cDNA using *B. amyloliquifaciens* RNA as template in reactions as described below.

The primer (500 ng) was mixed with the RNA (5  $\mu$ g) in 10  $\mu$ l of water and heated at 100°C for 1 min. The reaction was then made up to 20  $\mu$ l in 50 mM Tris-Cl, pH 8.0, 50 mM KCl, 7 mM MgCl<sub>2</sub>, 1 mM DTT, 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 25  $\mu$ M dCTP, 100  $\mu$ Ci [<sup>32</sup>P]-dCTP (2000 Ci/mmol), and 1 unit of reverse transcriptase, and incubated for 3 h at 37°C. For analytical purposes, 2  $\mu$ l of the reaction was electrophoresed on a 6% polyacrylamide, 7 M urea DNA sequencing gel as described elsewhere. The remainder of the samples were stored at -20°C and boiled 5 min just prior to use in hybridisation experiments.

(d) Preparation of strand-specific M 13 probes

Single stranded M 13 recombinant DNA prepared as described in the M 13 cloning and DNA sequencing section, was used to prepared [<sup>32</sup>P]-labelled DNA probes using the 5' primer essentially as described by Hu and Messing (1982).

The 5' primer (17-mer) was annealed to the M 13 single stranded recombinant DNA template in a 7  $\mu$ l reaction containing 1  $\mu$ l of primer (2.5 ng), 2  $\mu$ l of M 13 ss DNA preparation (about 1  $\mu$ g), 1  $\mu$ l of 10 X HIN buffer (0.1 M Tris-Cl, pH 7.4,

0.5 M NaCl, 0.1 M MgCl<sub>2</sub>) and 3 µl of water. The mix was boiled 3 min and allowed to cool slowly to room temperature over 45 min. To the annealing mix was added 1 µl of 10 mM DTT, 100 µCi of [<sup>32</sup>P]-dCTP (2000 Ci/mmol), 1 µl of nucleotide solution (0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP) and 1 µl (2 units) of Klenow fragment of DNA polymerase. The reaction was incubated at 15°C for 90 min and 1 µl of 0.25 M EDTA added to stop the reaction. The reaction was adjusted to 50 µl in 50 mM Tris-Cl, pH 8.0 and extracted with buffer saturated phenol:chloroform 1:1. Free label was removed by passage through a G-50 column and the fractions containing [<sup>32</sup>P]-labelled DNA pooled and used directly in hybridisation experiments.

#### HYBRIDISATIONS WITH [<sup>32</sup>P]-LABELLED DNA PROBES

Hybridisation of the various [<sup>32</sup>P]-cDNA probes used throughout this thesis with nitrocellulose filters prepared with DNA or RNA, or colony filters, was carried out essentially as described by Wahl *et al.* (1979) as detailed below.

The filters were prehybridised in a solution of 5 X SSC, 5 X Denhardt's solution, 50% formamide, 1% glycine, 50 mM KPO<sub>4</sub>, pH 7.4, and 200 µg/ml boiled, sonicated calf thymus DNA for 3 h at 42°C. Hybridisation was carried out in the same solution except that the DNA was at 50 µg/ml and Dextran sulphate added at 10%, with approximately 10<sup>6</sup> cpm of probe per filter, overnight at 42°C. Filters were washed (unless otherwise stated in the figure legends) in 2 X SSC, 0.1% SDS at room temperature for 1 h with 4 changes of wash solution, and then for 30 min in 0.2 X SSC, 0.1% SDS at 65°C, with two changes of wash solution, omitting the SDS in

the final wash. Unless otherwise stated, the washed, dried nitrocellulose filters were placed in contact with X-ray film, and exposed at  $-80^{\circ}\text{C}$  in the presence of an intensifying screen.

#### PREPARATION OF [ $^{32}\text{P}$ ]-PHOSPHORYLATED OLIGONUCLEOTIDE HYBRIDISATION PROBES

##### (a) [ $^{32}\text{P}$ ]-phosphorylation of oligonucleotides

The oligonucleotide mixtures described in Figure 5.1 were supplied free of the 5' terminal phosphate group and were labelled at the 5' end by incubation with  $\gamma$ -[ $^{32}\text{P}$ ]-ATP (2000 Ci/mmol) and T4 polynucleotide kinase as described below.

0.5  $\mu\text{g}$  of oligonucleotide was [ $^{32}\text{P}$ ]-phosphorylated in a 10  $\mu\text{l}$  reaction containing 50 mM Tris-HCl, pH 9.0, 10 mM  $\text{MgCl}_2$ , 10 mM DTT, 100  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ]-ATP and 1 unit of T4 polynucleotide kinase (added last). The reaction was incubated at  $37^{\circ}\text{C}$  for 45 min.

##### (b) Separation of the oligonucleotide from free label

To separate the oligonucleotide from unincorporated [ $\gamma$ - $^{32}\text{P}$ ]-ATP, 10  $\mu\text{l}$  of formamide loading solution (deionized formamide, 0.01% [w/v] bromophenol blue, 0.01% [w/v] xylene cyanol, 0.1 mM EDTA, pH 8.0) was added to the reaction after incubation and the sample electrophoresed on a 20% acrylamide gel (20 cm x 40 cm x 0.5 mm) in TBE (50 mM Tris-borate, pH 8.3, 1 mM EDTA) containing 7 M Urea at 15 mA until the tracker dye was approximately 15 cm into the gel. The radioactive oligonucleotide band was localised by autoradiography (30 sec) cut out and eluted from the gel overnight at  $37^{\circ}\text{C}$  in 400  $\mu\text{l}$  of elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1%

SDS) and ethanol precipitated using 5 µg of *E. coli* tRNA's as carrier. Just prior to use as probe this material was pelleted by centrifugation (10 min in the Eppendorf microfuge) washed in 70% (w/v) ethanol and then in absolute ethanol and finally dried under vacuum. The [<sup>32</sup>P]-phosphorylated oligonucleotide was then dissolved in the appropriate solution for use in hybridisation experiments.

(c) Sequencing of oligonucleotides

Prior to use in hybridisation experiments the sequences of the 14-mer mixtures described in Figure 5.1 were prepared as in (a) and (b) above and checked by sequencing using the chemical cleavage procedures of Maxam & Gilbert (1980), as modified by Banaszuk *et al.* (1983), and electrophoresis on a 20% polyacrylamide gel (20 cm x 40 cm x 0.5 mm) in TBE (50 mM Tris-borate, pH 8.3, 1 mM EDTA) containing 7 M Urea.

USE OF [<sup>32</sup>P]-PHOSPHORYLATED OLIGONUCLEOTIDES AS HYBRIDISATION

PROBES

Nitrocellulose filters were probed with [<sup>32</sup>P]-phosphorylated 14-mers 1 or 2 (Section 5.4) using a modification of the procedure of Wallace *et al.* (1981) as described below.

Prehybridisation was carried out in 6 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M Na citrate), 1% (w/v) glycine, 5 X Denhardt's solution (50 X = 1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA), 200 µg/ml of sonicated, boiled salmon sperm DNA for 2 h at 65°C followed by 2 h at 41°C. Hybridisation was carried out as above in fresh solution composed as above with the addition of 10% (w/v) Dextran sulphate and the appropriate [<sup>32</sup>P]-phosphorylated

oligonucleotide probe (approximately 0.2  $\mu\text{g}$ ,  $10^8$  cpm/microgram). Hybridisation was carried out overnight at 30°C. The filters were washed 3 times for 15 min each in 6 X SSC with 0.1% (w/v) SDS in the first 2 washes, air dried and autoradiographed at 4°C overnight.

#### SUBCLONING INTO M 13 PHAGE VECTORS AND DNA SEQUENCE ANALYSIS

Restriction fragments of DNA to be sequenced were subcloned into M 13 phage vectors (Winter, 1980; Messing & Vieira 1982) and sequenced using the dideoxy chain termination reactions of Sanger *et al.* (1977), as described below.

##### (a) Preparation of M 13 replicative form DNA.

To 3 mls of 0.7% agar at 45°C is added 30  $\mu\text{l}$  of BCIG, (5-bromo-4-chloro-3-indoyl-beta-galactaside, 20 mg/ml in dimethylformamide), 20  $\mu\text{l}$  of IPTG (isopropyl-beta-D-thio-galactopyranoside, 20 mg/ml in water) 0.2 ml of exponential (A600 = 0.6) JM101 culture and 0.1 ml of diluted M 13 phage (enough to give about 200 pfu). This mixture was poured on a minimal (+ glucose) plate and incubated at 37°C for 9 h.

A blue plaque was selected, toothpicked into 1 ml of 2 X TY broth and grown with shaking for 6 h. Meanwhile a 10 ml culture of JM101 from a single colony on a minimal glucose plate was grown to an A600 of 0.5, and added to 1 litre of 2 X TY. When the A600 of this culture reached 0.5, the 1 ml of phage solution was added and grown for 4 h.

Replicative form M 13 DNA was prepared from pelleted cells as follows. Cells were resuspended in 4 mls of 25 mM Tris-Cl, pH 8.0, 10 mM EDTA, 15% sucrose and 2 mg/ml lysosyme, and incubated on ice for 30 min. 8 mls of 0.2 M NaOH, 1% SDS



was added, gently mixed and left on ice for 10 min. The solution was neutralised by addition of 5 mls of 3 M Na-acetate, pH 4.6, and incubated on ice for a further 40 min. After centrifugation, (20,000 g for 15 min), the supernatant was carefully removed, 50  $\mu$ l of 1 mg/ml RNA'se A was added and incubated at 37°C for 20 min. The solution was extracted twice with an equal volume of buffer-saturated phenol:chloroform 1:1, and ethanol precipitated. The pellet was resuspended in 1.6 mls of water and the DNA precipitated by the addition of 0.4 ml of 4 M NaCl, 2 mls of 13% PEG (polyethyleneglycol) and standing on ice for 1 h. The DNA was collected by centrifugation, (20,000 g for 10 min), the PEG supernatant carefully removed and the pellet was finally washed in 70% ethanol and dried under vacuum.

(b) Preparation of M 13 vectors

M 13 vectors (from mp83 or mp93 replicative form DNA isolated as above) were prepared using the appropriate restriction enzyme(s) as described previously for plasmid pBR322 vectors. M 13 vector DNA was stored for use at 20 ng/ $\mu$ l.

(c) Ligation and transformation

Restriction fragments were cloned into the appropriate M 13 vector using a 3-fold molar excess of DNA over linear vector DNA in ligation reactions. A typical ligation reaction contained 1  $\mu$ l of DNA fragments, 1  $\mu$ l of 10 mM rATP, 1  $\mu$ l of 10 X C (0.5 M Tris-Cl, pH 7.5, 0.1 M MgCl<sub>2</sub>, 10 mM DTT), 5  $\mu$ l of water and 1  $\mu$ l of T4 DNA ligase, (0.5 units/ $\mu$ l). Reactions were incubated at 4°C for from 12 - 24 h. A control ligation with no added DNA fragments was

included to determine background transformation levels.

Competent cells were prepared by growing JM101 to an A600 of 0.6 in 2 X TY broth, harvesting by centrifugation (500 g, 5 min, 4°C) and resuspending in 1/20th original volume of freshly prepared 50 mM CaCl<sub>2</sub>. Cells were used after storage at 4°C for at least 2 h, and up to 3 days.

One fifth (2 µl) of a ligation mix was added to 0.2 ml of competent JM101 cells and kept on ice for 40 min. The cells were heat shocked at 42°C for 2 min and then added to 3 ml of 0.7% agar containing 20 µl BCIG (10 mg/ml), 20 µl IPTG (10 mg/ml) and 0.2 ml of exponential JM101 (A600 ~ 0.5). The mixture was plated on minimal (+ glucose) agar plates and grown for 9 - 12 h at 37°C.

(d) Preparation of template DNA for sequencing

Recombinant plaques were toothpicked into 1 ml of 2 X TY broth containing 2 µl of overnight JM101 culture and grown with shaking for 5 h at 37°C. Cells were pelleted by centrifugation in an Eppendorf centrifuge for 10 min, the supernatant removed carefully into a fresh tube and recentrifuged. To each supernatant was added 0.2 ml of 2.5 M NaCl, 20% PEG 6000, and after leaving at room temperature for 15 min, the phage pellet was collected by centrifugation for 5 min. After removal of all the supernatant, the pellet was resuspended in 0.1 ml of 10 mM Tris-Cl, pH 8.0, 0.1 mM EDTA and extracted with an equal volume of buffer saturated phenol. The aqueous phase was re-extracted twice with 0.5 ml of diethyl ether and ethanol precipitated. The phage DNA was collected by centrifugation, washed in 70% ethanol, 95% ethanol and finally dried under vacuum and dissolved in 25 µl of 10 mM Tris-Cl, pH 8.0, 0.1 mM

EDTA and stored at  $-20^{\circ}\text{C}$ .

(e) Complementarity testing of single-stranded (ss) M 13 recombinants

To determine which strand of a particular sub-cloned DNA fragment was present in a single-stranded M 13 recombinant (ss M 13 clone), hybridisation analysis was carried out using an arbitrarily selected, or previously sequenced ss M 13 clone as a reference. 2  $\mu\text{l}$  of ss M 13 clone DNA to be tested was added to 2  $\mu\text{l}$  of reference DNA with 4  $\mu\text{l}$  of 10 X HIN buffer (0.1 M Tris-Cl, pH 7.4, 0.1 M  $\text{MgCl}_2$ , 0.5 M NaCl) and 2  $\mu\text{l}$  of 5 X M 13 loading solution (50% glycerol, 1% SDS, 0.2 M EDTA, 0.2% bromophenol blue) and incubated at  $65^{\circ}\text{C}$  for 1 h. The samples were electrophoresed as described previously on a 1% agarose minigel, with 2 ml of reference DNA as a marker. The DNA was visualised after ethidium bromide staining. Single-stranded M 13 clones with inserts identical to the reference clone co-migrate with the reference, whereas clones with the complementary strand are retarded as they have hybridised to the reference, thereby doubling their molecular weight.

(f) Dideoxy chain termination DNA sequencing procedures

DNA sequence analysis of M 13 single-stranded DNA was carried out essentially as described by Sanger *et al.* (1977).

(1) Hybridisation: 2.5 ng of universal primer (17-mer) was annealed to 8  $\mu\text{l}$  of the template DNA preparation (about 1  $\mu\text{g}$ ) in a 10  $\mu\text{l}$  reaction containing 10 mM Tris-Cl, pH 7.4, 10 mM  $\text{MgCl}_2$ , 50 mM NaCl by boiling for 3 min and allowing the reaction mix to cool slowly over 1 h.

(2) Polymerisation: 4  $\mu\text{l}$  of  $\alpha$ [ $^{32}\text{P}$ ]-dATP (approximately

16  $\mu\text{Ci}$ ) was lyophilized, the hybridisation mixture added, 1  $\mu\text{l}$  of 10 mM DTT added and finally 1  $\mu\text{l}$  of Klenow fragment (1 unit) was added, and vortexed to resuspend the label. 2.5  $\mu\text{l}$  of this solution was added immediately to each of 4 tubes containing 2  $\mu\text{l}$  of the appropriate  $\text{N}^\circ$ -ddNTP work solution (freshly made from stock concentrates, see details below). After mixing, samples were incubated at 37°C for 15 min, 1  $\mu\text{l}$  of dATP chase (500  $\mu\text{M}$  dATP in 5 mM Tris-Cl, pH 8.0, 0.1 mM EDTA) added and incubation continued for a further 15 min at 37°C. 5  $\mu\text{l}$  of formamide loading buffer (formamide, deionised with mixed bed resin, 0.1% bromophenol blue, 0.1% zylene cyanol and EDTA to 20 mM) was added to each tube, the samples boiled for 3 min and then analysed on a sequencing gel (see below).

The  $\text{N}^\circ$ -ddNTP work solutions were composed as follows: A-work solution was 0.4 mM ddATP:A $^\circ$  (200  $\mu\text{M}$  dTTP, 200  $\mu\text{M}$  dCTP, 200  $\mu\text{M}$  dGTP, 5 mM Tris-Cl, pH 8.0), 1:1; C-work solution was 0.2 mM ddCTP:C $^\circ$  (200  $\mu\text{M}$  dTTP, 10  $\mu\text{M}$  dCTP, 200  $\mu\text{M}$  dGTP, 5 mM Tris-Cl, pH 8.0), 1:1; G-work solution was 0.3 mM ddGTP:G $^\circ$  (200  $\mu\text{M}$  dTTP, 200  $\mu\text{M}$  dCTP, 10  $\mu\text{M}$  dGTP, 5 mM Tris-Cl, pH 8.0), 1:1; T-work solution was 0.1 mM ddTTP:T $^\circ$  (10  $\mu\text{M}$  dTTP, 200  $\mu\text{M}$  dCTP, 200  $\mu\text{M}$  dGTP, 5 mM Tris-Cl, pH 8.0), 1:1.

(3) DNA sequencing gels: 1  $\mu\text{l}$  of each sample was electrophoresed on a 6% polyacrylamide, 7 M urea gel (20 cm x 40 cm x 0.2 mm) in TBE buffer (90 mM Tris-borate, pH 8.3, 2.5 mM EDTA). The gels were pre-electrophoresed for 45 min prior to loading the samples. Electrophoresis was at 1200 Volts until the tracker dyes had migrated the desired distance.

After electrophoresis the gels were fixed 10 min in 10% acetic acid, washed with several litres of water to remove the urea and dried. Autoradiography was generally carried out overnight at 4°C.

#### CONTAINMENT FACILITIES

All work involving recombinant DNA was carried out under C1/EK1 containment conditions for work involving viable organisms and Co containment conditions for work not involving viable organisms, as defined and approved by the Australian Academy of Science Committee on Recombinant DNA and by the University Council of the University of Adelaide.

#### GENERAL METHODS

##### (1) Cell densities

Cell densities were determined by measurement of the absorbance of a cell suspension (or a 1/20 dilution thereof in physiological saline) at 600 nm in a 1 cm cuvette using a Hitachi Model 101 Spectrophotometer.

##### (2) Protein Estimations

Estimates of total protein were carried out according to the method of Lowry (1951), using bovine serum albumin (BSA) as a standard.

##### (3) RNA and DNA Estimations

RNA and DNA concentrations were estimated spectrophotometrically assuming that one A260 unit equals 40 µg/ml for RNA or 50 µg/ml for DNA.

CHAPTER THREE

ISOLATION AND PARTIAL PURIFICATION OF  
PUTATIVE ALKALINE PROTEASE mRNA FROM  
*B. AMYLOLIQUEFACIENS*

### 3.1 INTRODUCTION

Our current understanding of the processes of extra-cellular enzyme production by *B.amyloliquefaciens* was discussed in the introductory chapter of this thesis. In particular, extracellular protease production by this organism has been the subject of a recent study in this laboratory (O'Connor, 1978, Love, 1981). As mentioned previously, as a result of this work, it seemed that a study of alkaline protease production at the molecular level was the most promising approach. The aim was to isolate the gene for the alkaline protease to enable direct hybridisation assay studies of protease messenger RNA production to be undertaken.

With these considerations in mind, the aims of the work described in this chapter were two-fold. Firstly, to prepare RNA containing mRNA for the alkaline protease as shown by translating total RNA *in vitro* and identifying alkaline protease amongst the translational products; and secondly, to partially purify this messenger RNA to permit synthesis of a cDNA probe for use in isolation of the alkaline protease gene.

Translation of mRNA isolated from *B. amyloliquefaciens* has been attempted in several *in vitro* systems derived from both prokaryotes and eukaryotes in this laboratory (O'Connor, 1978 and Bawden, 1979). This work was inconclusive firstly because for reasons discussed below, identification of proteases in mRNA *in vitro* translational products was difficult, and secondly because attention was focused on neutral protease. Since the work of Love (1981) showed that in the apparent accumulation of mRNA, alkaline protease mRNA predominated, the

latter protease was the one to concentrate on (as has already been mentioned in the introduction).

Translation of an mRNA for protease in an *in vitro* system raises several potential problems. Firstly, synthesis of an active protease, should it occur in a cell-free system may be detrimental to that system. Secondly, possible breakdown of the synthesised protease by autodigestion may render the identification of translation products difficult. Possibly as a result of these factors and/or the high level of RNAase present in cell homogenates of *B. amyloliquefaciens* or *B. subtilis* attempts to translate alkaline protease mRNA in such homologous systems were unsuccessful, (O'Connor, 1978; Bawden, 1979). This was disappointing since it seemed that if the mRNA reserve pool had "special" translational requirements those requirements were most likely to be met by homologous translational systems.

A cell-free translation system derived from *E. coli* was therefore tried essentially as described by Zubay (1973) and optimized for the translation of mRNA isolated from *B. amyloliquefaciens* (Bawden, 1979). This *in vitro* system proved capable of translating total RNA from *B. amyloliquefaciens* into a broad range of translational products.

This chapter describes the use of this cell-free system and the identification of translation products, synthesised under the direction of RNA isolated from *B. amyloliquefaciens*. Also described is the partial purification of the mRNA for these translation products, for use as a probe in the isolation of the alkaline protease gene.

In summary, my initial approach was to isolate total



RNA from *B. amyloliquefaciens*, translate it in an *in vitro* system derived from *E. coli*, and by various means, attempt to identify translation products as related to the alkaline protease of this organism. (The term "related" to alkaline protease" is used to indicate either a precursor protein or a degradation product of the protease).

### 3.2 PREPARATION OF THE *E. COLI* CELL-FREE TRANSLATION SYSTEM

The details of the preparation and components of the cell-free system derived from *E. coli* strain MRE 600 are given in Methods, Chapter 2. The S 30 extracts used throughout this thesis were prepared as described by Bawden (1979). Each extract was optimized for the various ion and other requirements in preliminary experiments. In general, each 50  $\mu$ l translation assay was composed of the components described in Chapter 2, with additions where appropriate as described in the figure legends, and the reaction mix incubated for twenty minutes at 37°C.

An important modification, shown in preliminary experiments to significantly reduce the level of background (no added RNA) incorporation in the translation assays was micrococcal nuclease treatment of the S 30 extract just prior to use in the translation system. This treatment was carried out essentially as described by Bottomley & Whitfeld (1979). In control experiments various levels of nuclease, and various times of incubation were tried to determine the most appropriate level of nuclease treatment in this system. The best protocol for nuclease treatment arrived at for use in all

future experiments is detailed in Methods, Chapter 2.

### 3.3 ISOLATION OF TOTAL RNA FROM *B. AMYLOLIQUEFACIENS*

A procedure for the extraction of RNA from *B. amyloliquefaciens* was developed by O'Connor (1978). It was found in previous attempts (Both, 1973) that the RNA of this organism was subject to extensive degradation during normal phenol extraction procedures, perhaps due to the high levels of ribonuclease produced by the organism. The procedure developed by O'Connor largely overcame this problem, yielding intact cellular RNA as judged by analysis on 4% polyacrylamide gels containing 98% (v/v) formamide. Briefly, in this procedure, cells were washed by resuspension in Tris buffer to remove extracellular ribonuclease, snap frozen in fresh buffer, lysed using the X - press apparatus at -25°C and RNA isolated from the lysate using an SDS/phenol extraction procedure. RNA isolated from *B. amyloliquefaciens* by the above procedure was translated well in the *E. coli* cell-free system.

It became clear from this work that isolation of intact RNA from this organism required the rapid and sustained inactivation of its associated ribonucleases. It was decided to attempt to improve the isolation procedure. Cox (1968) described a procedure for the isolation of RNA employing guanidium derivates as denaturing agents. Ullrich *et al.* (1977) successfully isolated intact RNA from the rat pancreas (a tissue rich in ribonucleases), employing guanidine thiocyanate as extractant (a strong denaturant and ribonuclease

inhibitor). Related procedures have become widely used in the isolation of RNA's from many sources.

A modification of the procedure of Ullrich *et al.* (1977) based on that of Brooker *et al.* (1980) using guanidine-hydrochloride was developed for the isolation of RNA from *B. amyloliquefaciens*. This procedure is given in Methods, Chapter 2.

In preliminary experiments this extraction procedure was compared with that of O'Connor (1978). Although both RNA preparations gave rise to the same product profile when translated in the *E. coli* cell-free system, for an equivalent amount of starting *B. amyloliquefaciens* cell culture, the new procedure yielded three times the amount of RNA. A typical preparation yielded approximately 6.0 mg of RNA from 240 ml of starting culture, providing sufficient material for preparative fractionation. RNA preparations from both procedures were compared by methylmercury-agarose gel electrophoresis (refer to Figure 3.2 to follow). The RNA's appear by staining to be essentially the same. This guanidine-hydrochloride extraction procedure was therefore used for all RNA preparations throughout the work described in this thesis.

#### 3.4 IN VITRO TRANSLATIONAL PRODUCTS DIRECTED BY B. AMYLOLIQUEFACIENS RNA IN THE E. COLI CELL-FREE SYSTEM.

A broad range of products result from translation of unfractionated RNA from *B. amyloliquefaciens* in the *E. coli* cell-free system, while in the control sample, the background incorporation is negligible, as a result of nuclease treatment of the *E. coli* S 30 just prior to use as discussed in Section

3.2 (This will be illustrated in Figure 3.1 to follow).

Preliminary experiments showed that the response of the *E. coli* cell-free system to added RNA (Bawden, 1979) was essentially linear in terms of [<sup>35</sup>S]-methionine incorporation into TCA-precipitable material between 0 and 150 µg of unfractionated *B. amyloliquefaciens* RNA per assay. The translation product profile was constant over this range of RNA addition. It is perhaps worth noting at this point, that the unfractionated RNA preparation is composed of approximately 90% ribosomal and transfer RNA's as estimated from stained profiles of the RNA on agarose gels. This will be discussed further in later sections of this chapter. In general in the work to follow, approximately 100 µg of this RNA per translation assay was used as indicated in the figure legends.

### 3.5 RNA ISOLATION FROM CELLS WITH A HIGH OR LOW APPARENT PROTEASE mRNA POOL

As described in the introduction the basis of this work was the initial observation that *B. amyloliquefaciens* cells harvested from culture were capable of prolonged rifampicin and actinomycin D-insensitive protease synthesis and that a reserve pool of mRNA (see the footnote below) was the hypothesis which best explained this observation.

In respect of the aim of the present work (that is, to provide an mRNA fraction suitable for synthesising a cDNA probe to enable isolation of the alkaline protease gene) it

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In the present work, use of the term "mRNA pool" is necessary for convenience, but does not imply that the existence of the pool is proven since this is a major purpose of the work. The qualification "apparent" is inserted to indicate this fact. It is emphasised that the term "mRNA pool" simply refers to the capacity of cells to synthesise protease in the presence of rifampicin or actinomycin D at levels which inhibit RNA synthesis greater than 95% and general protein synthesis completely.

would be useful to use an RNA preparation in which the level of mRNA for the protease is enriched.

It is possible to obtain cells which either have a zero level of apparent protease mRNA pool or alternatively a very high level. This is achieved by utilising the repressive effect of high concentrations of amino acids. Thus O'Connor *et al.* (1978) showed that incubation of cells for 75 min in medium containing a high level of amino acids exhausted the pool; transfer of such cells to low amino acids for 75 min caused a progressive accumulation of the apparent mRNA pool.

Therefore, using this property, RNA was isolated from *B. amyloliquefaciens* cells in which the apparent protease mRNA pool was minimal (that is after incubation of log phase cells for 75 min in high amino acids medium), and after transfer of these "exhausted" cells and a further incubation for say 60 min in low amino acids medium, after which time the apparent protease mRNA pool is approaching its maximal level.

It was of interest to determine whether these extremes of apparent protease mRNA pool size were demonstrable either in the profiles of RNA isolated from these two time points or in the translation products directed by these two RNA samples. Those studies required a means of fractionating the RNA and procedures for identification of *in vitro* translation products.

As a first step RNA was isolated from cells of *B. amyloliquefaciens* incubated for 75 min in high amino acids medium, and from cells incubated for a further 60 min in low amino acids medium as described in detail in Methods, Chapter 2. For convenience and brevity, these RNA samples are named 75 Haa RNA and 60 Laa RNA respectively.

The translation product profiles of these two RNA samples

are shown in Figure 3.1. It appears from this autoradiogram that the two samples are very similar in their protein coding capacity. The control sample (no added RNA, Track 1) shows negligible incorporation of label into protein products. Also shown in this photograph are the culture supernatant enzymes of *B. amyloliquefaciens*, labelled with [<sup>35</sup>S]-methionine as described in Methods and used as markers. The major enzymes are named and their approximate molecular weights given. The protein of central interest in this work is the extracellular alkaline protease which has a molecular weight of approximately 30,000 D. Of particular interest therefore are the prominent translation products in the region of this enzyme (arrowed).

The two RNA preparations were compared by analysis of samples on methylmercury/agarose gels run as described in Figure 3.2. This figure shows that in the RNA profile of 60 Laa RNA a range of bands below the 16S RNA appear more prominently than in the 75 Haa RNA sample. The possible significance of this is discussed later in this chapter.

### 3.6 TRANSLATION OF RNA ISOLATED FROM RIFAMPICIN TREATED CELLS OF *B. AMYLOLIQUEFACIENS*

In studies on the apparent mRNA pools for alkaline and neutral protease upon transfer of cells from high amino acids to low amino acids media, the work of Love (1981) had demonstrated that during rifampicin-insensitive protease production, the neutral and alkaline protease mRNA pools support enzyme synthesis for 30 and 60 - 80 min respectively. This suggested that the latter mRNA pool may last longer than the former. The production of protease after 20 min rifampicin preincubation represented *de novo* protein

FIGURE 3.1

TRANSLATION PRODUCTS DIRECTED BY *B. AMYLOLIQUE-*  
*FACIENS* UNFRACTIONATED RNA (75 HAA RNA AND 60  
LAA RNA SAMPLES) IN THE *E. COLI* CELL-FREE SYSTEM

*B. amyloliquefaciens* unfractionated RNA samples 75 Haa RNA and 60 Laa RNA (defined in text) isolated as described in Methods, Chapter 2 were translated in the *E. coli* cell-free system. Samples were electrophoresed on a 12.5% SDS-polyacrylamide gel (along with [<sup>35</sup>S]-methionine labelled *B. amyloliquefaciens* culture supernatant protein markers) and the gel autoradiographed after fluorography. Details of all preparations and reactions are provided in Methods, Chapter 2. The major products in the region of the alkaline protease are arrowed and the culture supernatant enzymes labelled.

TRACKS:

1. No added RNA - control products
2. 60 µg 75 Haa RNA translated
3. 80 µg 75 Haa RNA translated
4. *B. amyloliquefaciens* culture supernatant proteins
5. 60 µg 60 Laa RNA translated
6. 90 µg 60 Laa RNA translated.

Am	≡	α-amylase	Mol. wt.	≈	60,000 D
Ne	≡	neutral protease	Mol. wt.	≈	45,000 D
Al	≡	alkaline protease	Mol. wt.	≈	30,000 D

( TRACK BETWEEN 1 + 2  
NOT RELEVANT )

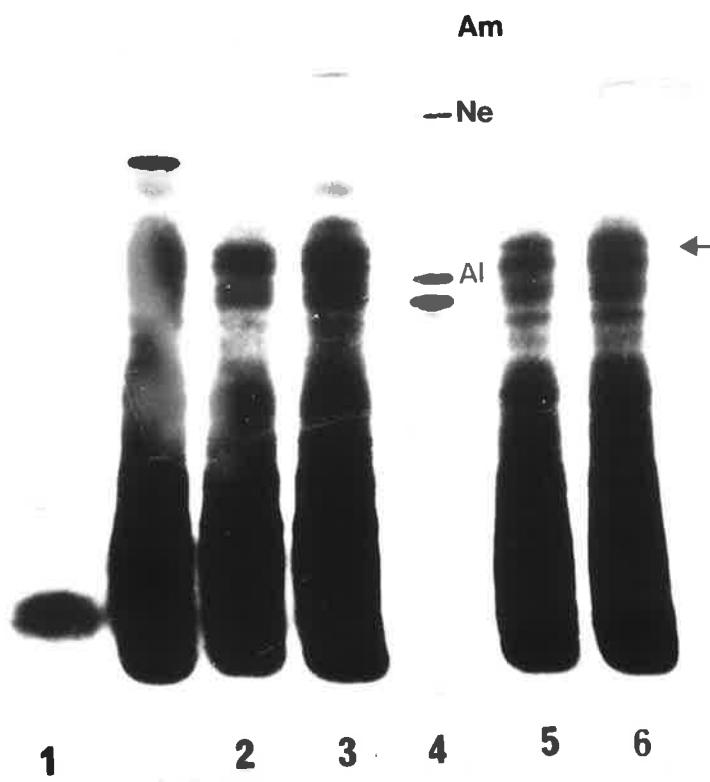




FIGURE 3.2

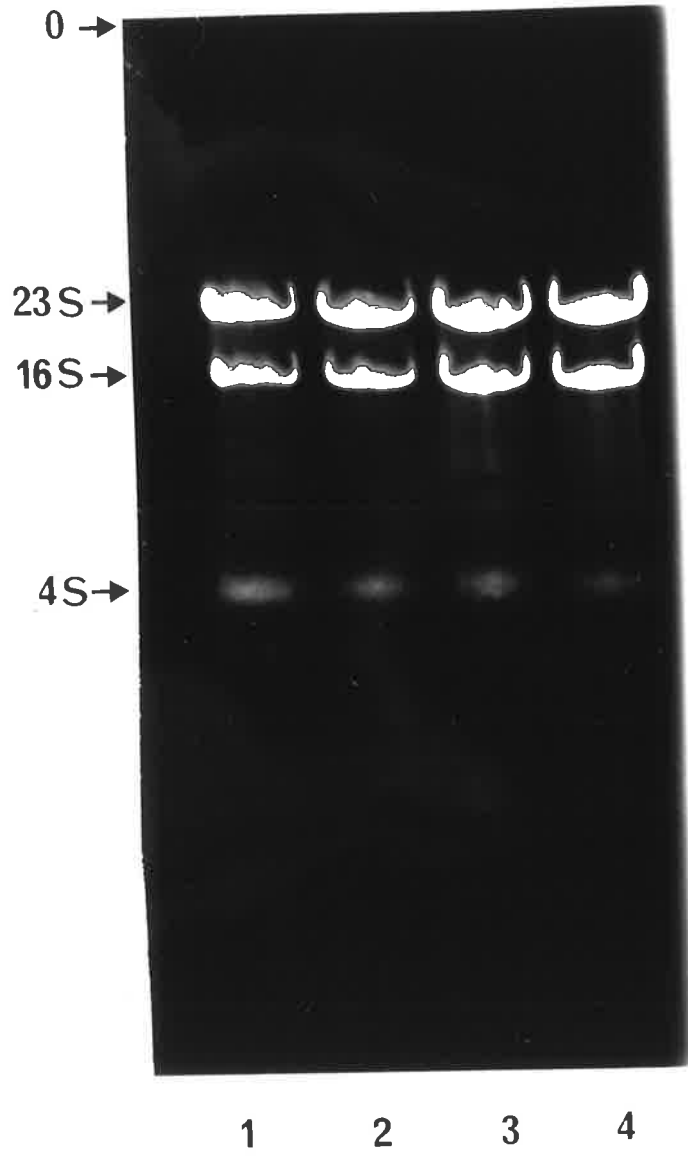
AGAROSE-METHYLMERCURY GEL ANALYSIS OF 75 HAA RNA  
AND 60 LAA RNA OF *B. AMYLOLIQUEFACIENS* (COMPARISON  
OF SDS/PHENOL AND GUANIDINE HYDROCHLORIDE RNA  
ISOLATION PROCEDURES)

10  $\mu$ g of each RNA sample isolated using either the SDS/phenol or guanidine HCl extraction procedures was electrophoresed on a 1% agarose gel containing 5 mM methylmercury hydroxide as described in Methods, Chapter 2.

TRACKS:

- |    |             |                         |
|----|-------------|-------------------------|
| 1. | 75 Haa RNA) | SDS/phenol procedure    |
| 2. | 60 Laa RNA) |                         |
| 3. | 75 Haa RNA) | guanidine-HCl procedure |
| 4. | 60 Laa RNA) |                         |

The 23 S rRNA, 16 S rRNA and 4 S RNA are indicated.



synthesis and was shown to be confined only to alkaline protease. Love (1981) also showed that incubation of cells with a maximum capacity for rifampicin-insensitive alkaline protease production in the presence of rifampicin for 20 min results in alkaline protease synthesis representing about 24% of total protein synthesis.

With these findings in mind, it was important to determine whether this apparent long-lived pool of alkaline protease mRNA could be demonstrated to exist by *in vitro* translation of RNA from cells optimised for the proposed pool and treated with rifampicin for 20 min to deplete other less stable mRNA's (see footnote below). It might be expected that translation of mRNA for alkaline protease would predominate when RNA isolated from cells optimised for the proposed mRNA pool (that is log phase cells of *B. amyloliquefaciens* incubated for 75 min in Haa medium followed by incubation for 60 min in Laa medium) and then treated for 20 min with rifampicin, was translated in the *E. coli* cell-free system.

Cells were optimised as above, and then incubated for a further 20 min in low amino acids medium containing 0.1 µg/ml rifampicin, and the RNA isolated as described in Methods, Chapter 2. This level of rifampicin was shown in control experiments to result in almost complete (greater than 95%)

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For clarity it perhaps needs to be pointed out that the essential concept in the mRNA pool hypothesis is that there is excess protease mRNA in the sense that this is not rate limiting in the synthesis of protease. Whether the prolonged synthesis of protease in the absence of transcription is due to there being such large amounts that the synthesis sites are saturated even after many half life decay periods or whether the mRNA is more stable or to a combination of these is not speculated upon.

inhibition of  $^3\text{H}$ -uracil incorporation into TCA precipitable material by washed cells (Love, 1981).

RNA was also isolated from a control sample of cells, incubated as above, but without the addition of rifampicin.

These two RNA samples were fractionated on sucrose gradients as described in Methods, Chapter 2 to obtain an enriched mRNA fraction and translated in the *E. coli* cell-free system. This fractionation of RNA will be discussed in detail later in this chapter. Figure 3.3 shows the result of this translation experiment.

The RNA from cells not treated with rifampicin translated to give the range of products seen in previous experiments. However, this range of products was drastically and uniformly depleted in the corresponding translation of RNA isolated from rifampicin treated cells.

This indicates that the messenger RNA's of *B. amyloliquifaciens* translated in this *in vitro* system all behave as typical, short half life prokaryote mRNA's. There appears to be no enrichment for translation products related to a stable mRNA. This raises the interesting possibility that the apparent pool of mRNA for alkaline protease is not in a form which is translatable in this *in vitro* system, and therefore, that the apparent mRNA pool may not be demonstrable by translation assay.

### 3.7 METHYLMERCURY TREATMENT OF THE RNA PRIOR TO TRANSLATION

One means by which an mRNA molecule may be rendered incapable of immediate translation is by the formation of secondary structure, giving a folded conformation in which

FIGURE 3.3

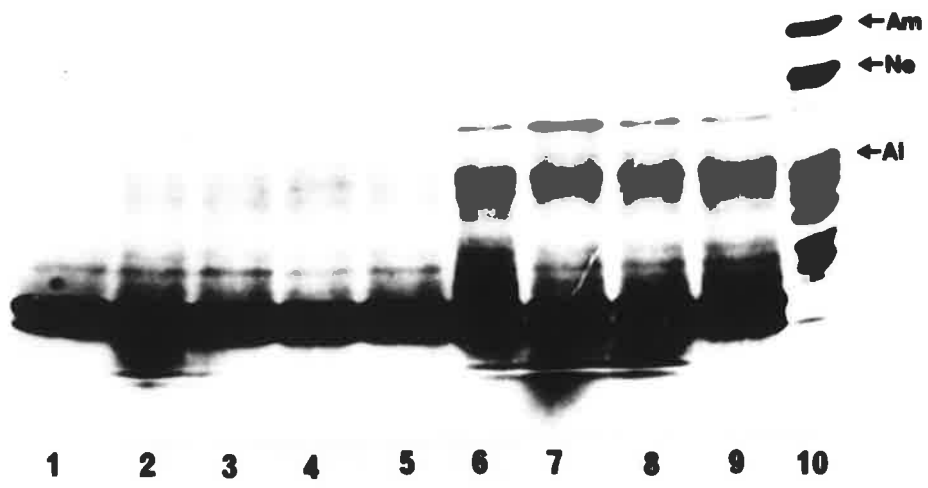
TRANSLATION PRODUCTS DIRECTED BY RNA ISOLATED  
FROM *B. AMYLOLIQUEFACIENS* CELLS, WITH OR WITHOUT  
RIFAMPICIN TREATMENT, IN THE *E. COLI* CELL-FREE  
SYSTEM

RNA was isolated from *B. amyloliquefaciens* cells as described in Methods, Chapter 2 except that incubation in low amino acids medium was carried out for an extra 20 min in the presence or absence of rifampicin (added to a final concentration of 0.1 µg/ml). The RNA was fractionated on sucrose gradients and 30 µg of each fraction translated in the *E. coli* cell-free system. Samples of the translation products were electrophoresed on a 12.5% polyacrylamide gel which was processed by fluorography and autoradiographed. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens* were used as markers. Details of all preparations and procedures are provided in Methods, Chapter 2.

TRACKS of translation products directed by:

1. No added RNA - control translation products
2. Gradient Fraction 1)
3. Gradient Fraction 2)      RNA from rifampicin treated cells.
4. Gradient Fraction 3)
5. Gradient Fraction 4)
6. Gradient Fraction 1)
7. Gradient Fraction 2)
8. Gradient Fraction 3)      RNA from control cells (not  
treated with rifampicin)
9. Gradient Fraction 4)
10. [<sup>35</sup>S]-methionine labelled *B. amyloliquefaciens* culture  
supernatant proteins.

Am ≡ α-amylase  
Ne ≡ neutral protease  
Al ≡ alkaline protease



the ribosome binding sites are not readily accessible. Payvar & Schimke (1979) have shown that the translation of certain mRNA's is enhanced by prior treatment of the mRNA with methylmercury, a strong denaturant which destroys secondary structure in nucleic acid.

To test whether translation of any mRNA's isolated from *B. amyloliquefaciens* were affected by methylmercury treatment, unfractionated RNA was treated with the denaturant just prior to translation. The details of this experiment are given in Figure 3.4.

Two RNA samples were treated with methylmercury. These were isolated from cells in which the apparent mRNA pool for protease was depleted (i.e. after incubation of cells for 75 min in high amino acids medium) and from cells in which the apparent pool was high (i.e., from cells incubated in high amino acids medium as above then for a further 60 min in low amino acids medium).

Figure 3.4 shows the results of translation of these two RNA samples with or without prior methylmercury treatment. As can be seen in the autoradiogram, methylmercury appears to have had no effect on the translation of either RNA sample. That is, no specific mRNA's appear to be enhanced in translation (as measured by the intensity of translation products) due to methylmercury treatment.

In this experiment, methylmercury was used at a final concentration of 5 mM. In further trials, increasing the concentration of methylmercury did not improve the previous result.

Therefore, it appears that the apparent mRNA pool for protease is not revealed in these translation assay experi-

FIGURE 3.4

TRANSLATION PRODUCTS DIRECTED BY 75 HAA RNA AND  
60 LAA RNA OF *B. AMYLOLIQUEFACIENS*, WITH OR  
WITHOUT PRIOR METHYLMERCURY TREATMENT OF THE RNA  
IN THE *E. coli* CELL-FREE SYSTEM

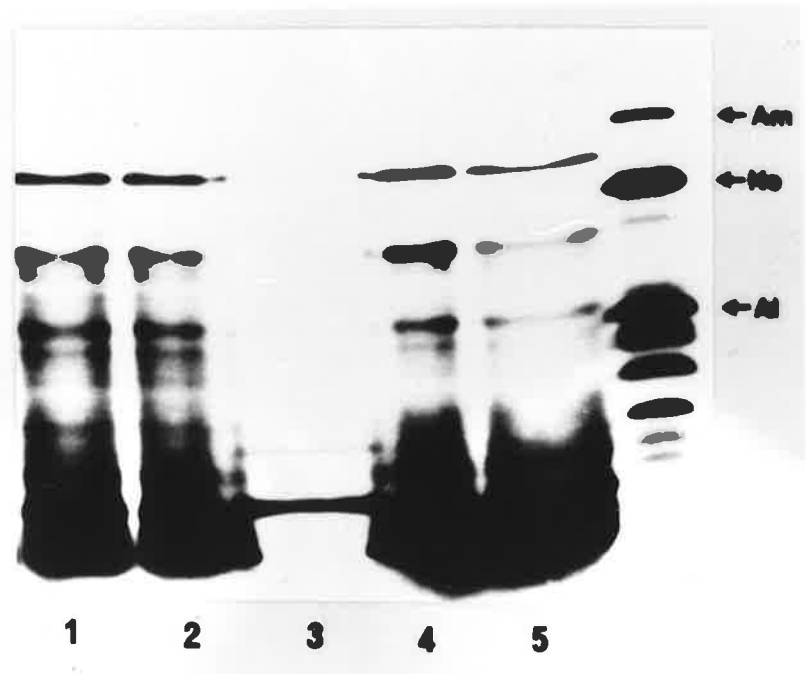
30 µg of each RNA sample (i.e., 75 Haa RNA or 60 Laa RNA) isolated as described in Methods, Chapter 2, was treated with methylmercury (at 5 mM, final concentration) for five minutes at room temperature just prior to translation in the *E. coli* cell-free system. Corresponding untreated samples were also translated. Samples of the translation products were electrophoresed on a 12.5% polyacrylamide gel which was processed by fluorography and autoradiographed. Details of all preparations and procedures are provided in Methods, Chapter 2. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens* were used as markers and the major enzymes are labelled.

TRACKS of translation products directed by:

1. 75 Haa RNA
2. 75 Haa RNA (methylmercury treated)
3. no added RNA (control products)
4. 60 Laa RNA
5. 60 Laa RNA (methylmercury treated)
6. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens*.

Am ≡ α-amylase  
Ne ≡ neutral protease  
Al ≡ alkaline protease





ments. If the pool really exists, then factors unique to the cytoplasm of *B. amyloliquefaciens* may be required for its translation. Another possible approach to look further at this would be to use a translation system incorporating cell free extracts of *B. amyloliquefaciens*. However, again the problem of ribonuclease emerges as cell extracts would need to be isolated using non-denaturing conditions to maintain the integrity of translation specific factors. This approach seems very complex and overly consumptive of time and effort.

Obviously, the approach of choice is to look at the apparent mRNA pool by hybridisation analysis using a gene probe.

### 3.8 PREPARATION OF AN ANTISERUM TO THE EXTRACELLULAR ALKALINE PROTEASE OF *B. AMYLOLIQUEFACIENS*

It appeared from the previous work that none of the translation products obtained by translation of RNA from *B. amyloliquefaciens* in the *E. coli* cell-free system could be shown to result from translation of mRNA's remaining after normal mRNA's had degraded. The possibilities have been discussed that the mRNA for the alkaline protease is not translatable in this system, or that translation products are rapidly broken down.

Immunoprecipitation is a useful tool in determining whether translation products synthesised in the *E. coli* cell-free system in response to mRNA from *B. amyloliquefaciens* are related to the secreted alkaline protease of this organism. This approach required first the preparation of an antiserum specific for this enzyme. Such an antiserum became available

for use at this stage of the work.

The culture supernatant alkaline protease of *B. amyloliquefaciens* has been purified to homogeneity (as judged by electrophoresis on SDS denaturing gels) in this laboratory (Love (1981) and O'Connor, personal communication). Samples of this preparation were used to raise antisera in a rabbit according to the regimen described in Methods, Chapter 2. Two rabbits were immunized and one showed a strong response in Ouchterlony immunodiffusion experiments, giving a single strong precipitation line when tested against the pure antigen. This antiserum was used in the following experiments without further purification.

As a further check of the specificity of this antiserum it was tested against the culture supernatant enzymes of *B. amyloliquefaciens* as detailed in Figure 3.5. The culture supernatant enzymes were labelled with [<sup>35</sup>S]-methionine and subjected to immunoprecipitation as described in Methods, Chapter 2, and in the figure legend, and then displayed on a polyacrylamide gel as shown.

The autoradiogram shows that the antiserum reacts very strongly with the native alkaline protease, and several lower molecular weight proteins which may be breakdown products of this enzyme. There is no cross-reaction with the  $\alpha$ -amylase but a very faint reaction with the neutral protease can be seen, (more prominently in a longer exposure of this gel). This may occur if the two proteases share some common minor antigenic determinants, or if the protease preparation used as antigen contained some breakdown products of the neutral protease.

## FIGURE 3.5

### IMMUNOPRECIPITATION OF *B. AMYLOLIQUEFACIENS*

### CULTURE SUPERNATANT PROTEINS WITH THE ALKALINE

### PROTEASE ANTISERUM

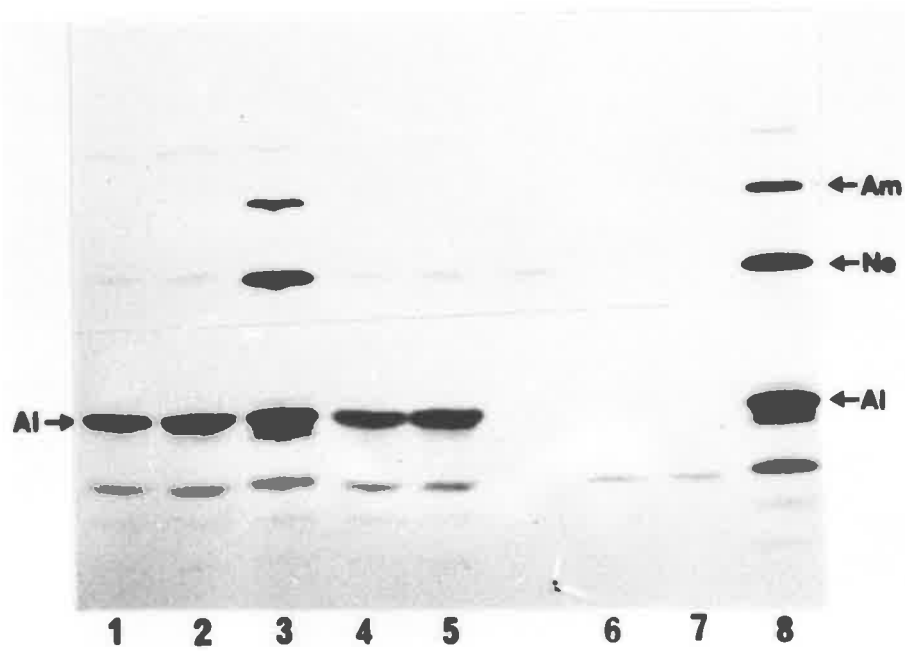
The culture supernatant proteins of *B. amyloliquefaciens* were labelled with [<sup>35</sup>S]-methionine as described in Methods, Chapter 2. 200 µl samples of labelled supernatant were treated as indicated below and immunoprecipitated using 10 µl or 20 µl of alkaline protease antiserum. The antiserum was prepared, and the immunoprecipitations carried out as described in Methods, Chapter 2. The samples were electrophoresed on a 12.5% polyacrylamide gel which was processed by fluorography and autoradiographed. Details of all preparations and procedures are provided in Methods, Chapter 2. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens* were used as markers and the major enzymes are labelled.

### TRACKS

1. 200 µl supernatant, 10 µl antiserum used
2. 200 µl supernatant, 20 µl antiserum used
3. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens*
4. as for 1 with PMSF at 2 mM included throughout all operations
5. as for 2 with PMSF
6. 200 µl supernatant with SDS at 2%, boiled for two min prior to immunoprecipitation, then as for 1.
7. 200 µl supernatant with SDS at 2%, boiled for two min prior to immunoprecipitation, then as for 2.
8. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens*

Am ≡ α-amylase  
Ne ≡ neutral protease  
Al ≡ alkaline protease

( TRACK BETWEEN 5 + 6  
NOT RELEVANT )



Two levels of antiserum were tried (10 & 20  $\mu$ l, Track 1 & 2 respectively). PMSF (at 2 mM) a serine protease inhibitor was included in the samples on Tracks 4 & 5, but appeared to have no effect. Tracks 6 & 7 show the result of boiling the samples for two minutes in the presence of SDS (2% [w/v]) prior to immunoprecipitation with the antiserum. This treatment has been shown by Matsuura *et al.* (1981) to enhance the reactivity of antigens with antisera in similar experiments. In this case however, the antiserum appears to react only with antigenic determinants on the untreated enzyme preparation. In any case, the high reactivity of this antisera makes it a useful tool in the identification of proteins related to this enzyme in the mixture of *in vitro* translation products synthesised in response to mRNA from *B. amyloliquefaciens*.

### 3.9 IMMUNOPRECIPITATION TRIALS USING THE ANTISERA TO ALKALINE PROTEASE

Immunoprecipitation of the translation products of *B. amyloliquefaciens* total RNA in the *E. coli* cell-free system was attempted using the procedure described in Methods, Chapter 2 and Figure 3.6. The autoradiogram presented here shows the results of immunoprecipitation from products synthesised in response to 75 Haa RNA or 60 Laa RNA. Several translation products are selectively precipitated by the alkaline protease antisera. Notable is the immunoprecipitation of the prominent translation product of slightly slower mobility than the culture supernatant alkaline protease

## FIGURE 3.6

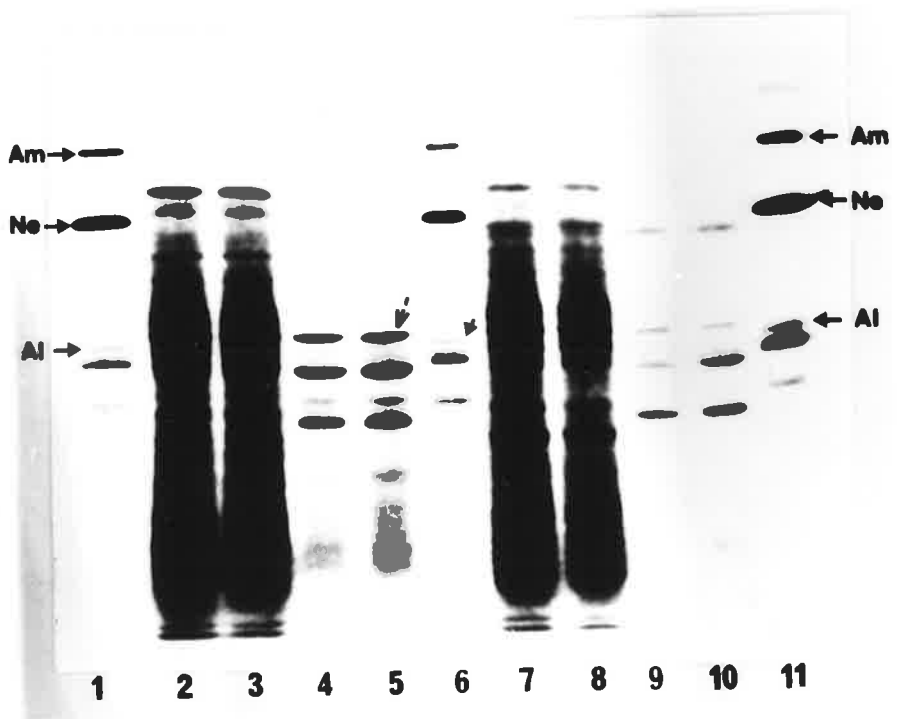
### IMMUNOPRECIPITATION OF TRANSLATION PRODUCTS DIRECTED BY 75 HAA RNA OR 60 LAA RNA IN THE *E. COLI* CELL-FREE SYSTEM, USING THE ALKALINE PROTEASE ANTISERUM

Samples (100  $\mu$ g) of 75 Haa RNA or 60 Laa RNA isolated from *B. amyloliquefaciens* cells as described in Methods, Chapter 2, were translated in the *E. coli* cell-free system. 10  $\mu$ l of each 50  $\mu$ l translation assay was prepared directly for electrophoresis, in the presence or absence of PMSF (2 mM). The remainder of each assay (40  $\mu$ l) was subject to immunoprecipitation using 20  $\mu$ l of alkaline protease antiserum, again in the presence or absence of PMSF (2 mM) throughout the procedure. The samples were electrophoresed on a 12.5% polyacrylamide gel which was processed by fluorography and autoradiographed. Details of all preparations and procedures are provided in Methods, Chapter 2. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens* were used as markers and the major enzymes are labelled.

#### TRACKS

1. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens*
2. 75 Haa RNA translation products
3. 75 Haa RNA PMSF (2 mM) present
4. 75 Haa RNA sample immunoprecipitated
5. as for 4. with PMSF (2 mM) present
6. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens*
7. 60 Laa RNA translation products
8. 60 Laa RNA translation PMSF (2 mM) present
9. 60 Laa RNA sample immunoprecipitated
10. as for 9. with PMSF (2 mM) present
11. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens*

Am  $\equiv$   $\alpha$ -amylase  
Ne  $\equiv$  neutral protease  
Al  $\equiv$  alkaline protease





(arrowed in Figure 3.6). The position of this product relative to the culture supernatant enzyme is consistent with it being a precursor product, not processed in the *in vitro* system. The lower molecular weight products may possibly be related also, as breakdown products.

In Figure 3.6 it can be seen that there is also a faintly precipitated product running below the neutral protease. This could conceivably be an incomplete neutral protease since it had been noted earlier that the antiserum weakly cross-reacted with the culture supernatant neutral protease. In control trials, the antiserum precipitated nothing from translations in which no RNA was added (ie. control translation products).

The strong immunoprecipitation of a translation product running just above the alkaline protease was a very encouraging result. However, an unexpected result was the finding that 60 Laa RNA gave rise to a weaker band of this product than that directed by 75 Haa RNA. From all the earlier studies the reverse would have been expected. In the total translation products, of the two RNA samples, there is no significant difference in intensity between the products of this mobility.

It had been noted in earlier work that the total products from 60 Laa RNA always appeared slightly "fuzzy" on an autoradiogram when compared to 75 Haa RNA. This problem was largely overcome by boiling samples immediately after translation in SDS-gel loading buffer as described in Methods, Chapter 2. The possibility arose that proteolytic breakdown of translation products was occurring, perhaps due to the

synthesis of an active protease during translation.

Love (1981) showed that the serine protease inhibitor PMSF, when added at 2 mM final concentration, completely eliminated alkaline protease activity in the culture supernatant of *B. amyloliquefaciens*. This inhibitor was tried at various levels in preliminary translation experiments, but appeared to have no effect on the quality of translation products. Levels above 5 mM interfered with the translation assay itself, resulting in drastically reduced incorporation of [<sup>35</sup>S]methionine into TCA precipitable material. PMSF has been shown by Sekar & Hageman (1979) to react with at least 14 different proteins in *B. subtilis* extracts and 15 in rabbit liver extracts, and is therefore to be used with caution in these types of experiments.

In early immunoprecipitation trials the discrepancy between the intensities of the immunoprecipitates of the two RNA samples was also noticed. It is possible that over the long time period of the immunoprecipitation reaction, significant breakdown of translation products may occur. Since in 60 Laa RNA we expect more mRNA for alkaline protease than in 75 Haa RNA, then translation of this RNA into active products may result in increased breakdown (also including autodigestion) in the 60 Laa RNA sample when compared to the 75 Haa RNA products. This could help to explain the "fuzziness" commented on previously and also the puzzling result of the immunoprecipitation trials.

Therefore, in the experiment described in Figure 3.6, in one set of samples PMSF was maintained throughout at 2 mM, final concentration. Again, this protease inhibitor appears

to have no effect at a concentration shown by Love (1981) to deal very effectively with the culture supernatant alkaline protease.

It is perhaps worthwhile to mention again here that the central aim of this work was to produce a probe suitable for use in isolation of the alkaline protease gene of *B. amyloliquefaciens*. We have, in the translation products of 75 Haa RNA, a prominent product related by immunoprecipitation to the alkaline protease, and of a size consistent with it being a precursor product of this enzyme. While according to the hypothesis discussed in Chapter 1 we expect significantly more mRNA for protease in the 60 Laa RNA sample, the *in vitro* work presented here does not positively indicate this, nor can we assume it to be the case.

The most reasonable approach then, to obtain a suitable probe for isolation of the gene of interest, was to partially purify mRNA for the appropriate translation product from 75 Haa RNA. While the capacity of log phase cells of *B. amyloliquefaciens* for rifampicin-insensitive protease production has been shown to be exhausted by incubation of cells for 75 min in high amino acids medium, a basal level of production always occurs, the mRNA for which appears to be translated in the *E. coli* cell-free system.

### 3.10 PEPTIDE MAPPING OF TRANSLATION PRODUCTS DIRECTED BY

#### *B. AMYLOLIQUEFACIENS* RNA

Prior to proceeding with fractionation of the RNA of *B. amyloliquefaciens*, the translation products in the region

of the culture supernatant alkaline protease were examined by peptide mapping using V<sub>8</sub> protease essentially as described by Cleveland *et al.* (1977). The mapping experiments were carried out by Dr. R. O'Connor in this laboratory. They involved excision of a section of the first dimension gel containing products in the region of the alkaline protease and digestion with V<sub>8</sub> protease in a second gel as described, (Bordier & Crettol-Jarvinen, 1979). [<sup>35</sup>S]-methionine labelled culture supernatant alkaline protease was subjected to the same procedure. The details are given in Figure 3.7. Indicated in the figure are the V<sub>8</sub> protease peptide maps for the alkaline protease and the major translation products in the size region of alkaline protease. It can be seen that a translation product is present (arrowed, Track 3) which when digested with V<sub>8</sub> protease, results in four peptides of the same size as those resulting when the supernatant alkaline protease is digested in the same manner (arrowed, Track 4). V<sub>8</sub> mapping directly of immunoprecipitated products was attempted but insufficient material was available to give a visible result.

This mapping result lends support for the presence of a translation product related to the alkaline protease, in the total products resulting from the translation in the *E. coli* cell-free system of RNA isolated from *B. amyloliquefaciens*.

The following work describes partial purification of the mRNA for this product for use as a probe in isolation of the alkaline protease gene.

### 3.11 SUCROSE GRADIENT FRACTIONATION OF *B. AMYLOLIQUEFACIENS* RNA

It was important to fractionate the desired mRNA as much as possible prior to use in the synthesis of a cDNA probe. Total RNA isolated from *B. amyloliquefaciens*, as was seen by

## FIGURE 3.7

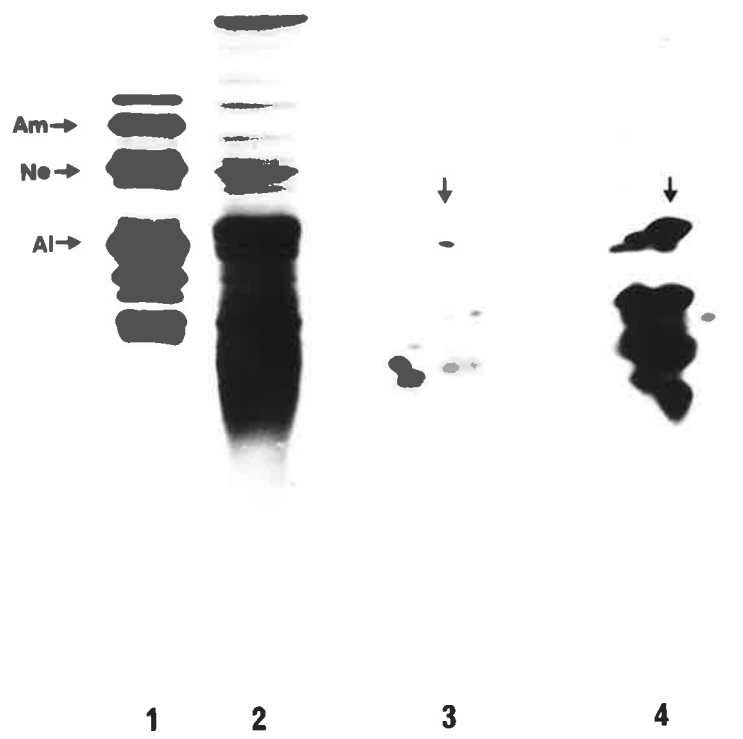
### V8 PEPTIDE MAPPING OF TRANSLATION PRODUCTS IN THE REGION OF THE SUPERNATANT ALKALINE PROTEASE

75 Haa RNA (100 µg) isolated as described in Methods, Chapter 2 was translated in the *E. coli* cell-free system and the products electrophoresed on a 12.5% polyacrylamide gel. Also electrophoresed was [<sup>35</sup>S]-methionine labelled culture supernatant marker proteins. Gel pieces containing proteins in the size region of alkaline protease were excised from this first dimension gel and mapped using V8 protease in a second gel (15% polyacrylamide) as described by Bordier & Crettol-Jarvinen (1979). This second gel, shown here, was processed by fluorography and autoradiographed.

#### TRACKS

1. [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens*
2. 75 Haa RNA total translation products
3. V8 mapping of translation products in the region of alkaline protease
4. V8 mapping of culture supernatant alkaline protease

Am ≡ α-amylase  
Ne ≡ neutral protease  
Al ≡ alkaline protease



gel analysis, is composed predominantly of ribosomal RNA's. It was obviously desirable to remove as much as possible of these RNA species.

RNA isolated from cells incubated for 75 min in high amino acids medium was chosen for fractionation because, as was discussed in Section 3.9, this RNA preparation was shown (paradoxically) to direct the most abundant synthesis of translation products demonstrable by immunoprecipitation to be related to the alkaline protease of *B. amyloliquefaciens*. The use of RNA isolated from cells incubated in high amino acids medium also provides the following advantage. Methyl-mercury gel analysis, of RNA preparations isolated over a time course of incubation in high amino acids followed by low amino acids medium, indicated that prolonged incubation in low amino acids medium resulted in extensive breakdown of the rRNA species into smaller molecular weight materials. This is consistent with the occurrence of a degree of "shutdown" of cellular functions in response to conditions of amino acid depletion, which does not occur during incubation in high amino acids medium. The use of 75 Haa RNA therefore minimizes the contamination of mRNA with rRNA breakdown products. Therefore in the work to follow, unless otherwise mentioned, the RNA isolated from cells incubated for 75 min in high amino acids medium was used.

The size of the mRNA required for the alkaline protease was calculated to give a Svedberg value of approximately 13S, (O'Connor, 1978). Gradient analysis of RNA isolated from cells at various stages during oscillation of the apparent protease mRNA pool carried out by O'Connor (1978) showed a 13S RNA peak, the size of which oscillated in concert with the apparent pool of mRNA. This region of the gradient was

therefore likely to contain the mRNA of interest, although as discussed previously, the changes in apparent pool size were not demonstrable by *in vitro* translation.

As a starting point the *B. amyloliquefaciens* RNA was fractionated using the same gradient system as that used by O'Connor (1978). In my hands this resulted in a typical profile as shown in Figure 3.8. This profile is somewhat different from those obtained in the previous work, and may reflect the different RNA preparation procedures used. Five broad RNA fractions were taken and RNA from each fraction translated in the *E. coli* cell-free system. Tracks 6, 7, 8 and 9 of Figure 3.3 show the translation products of four typical RNA fractions obtained using this sucrose gradient system. The results of this translation experiment indicated that mRNA's for the various products were broadly spread across the entire gradient.

This poor fractionation may reflect the fact that this gradient system was non-denaturing in character. Therefore a more stringent fractionation system was sought.

An alternative gradient system was tried which employs formamide as a denaturing agent. The gradients are run in 70% (v/v) formamide and a linear gradient of sucrose from 5% (w/v) to 25% (w/v) as described in Methods, Chapter 2. Figure 3.9 shows a typical profile of RNA run on this gradient system and fractionated as indicated. It is perhaps worth noting here that prior to loading on these gradients the RNA samples were denatured with methylmercury, as described in Methods, Chapter 2. This was shown in preliminary work to improve the fractionation, and was therefore carried out routinely.



FIGURE 3.8

SUCROSE GRADIENT OPTICAL DENSITY PROFILE OF

*B. AMYLOLIQUEFACIENS* 75 HAA RNA

75 Haa RNA was prepared and 200  $\mu$ g centrifuged on a 10-40% sucrose density gradient for 16 h, 4°C at 34,000 rpm and the gradient band fractionated as indicated. Details of all preparations and procedures are provided in Methods, Chapter 2. The optical density profile (254 nm) is shown.

Optical Density (254 nm)

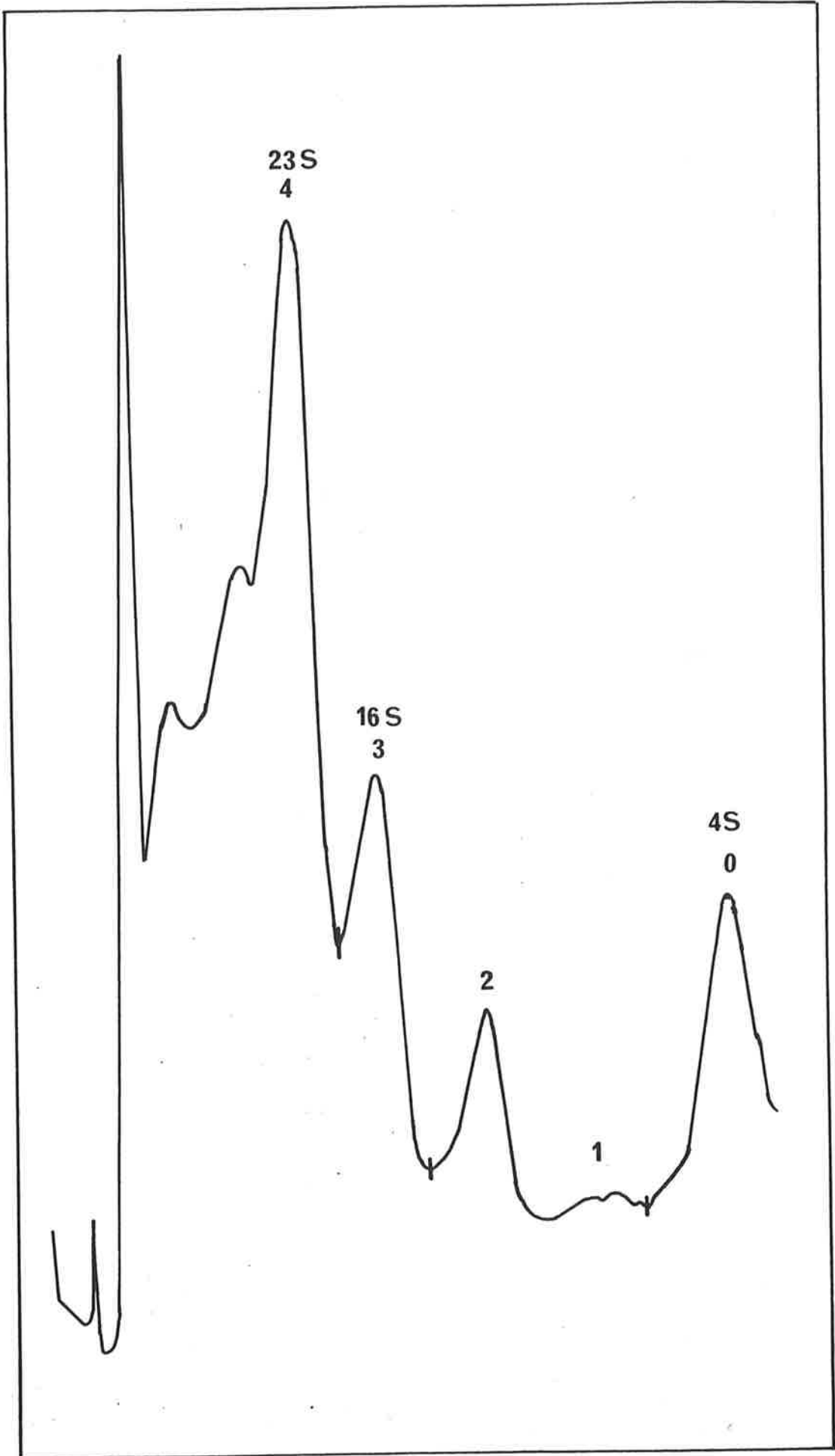


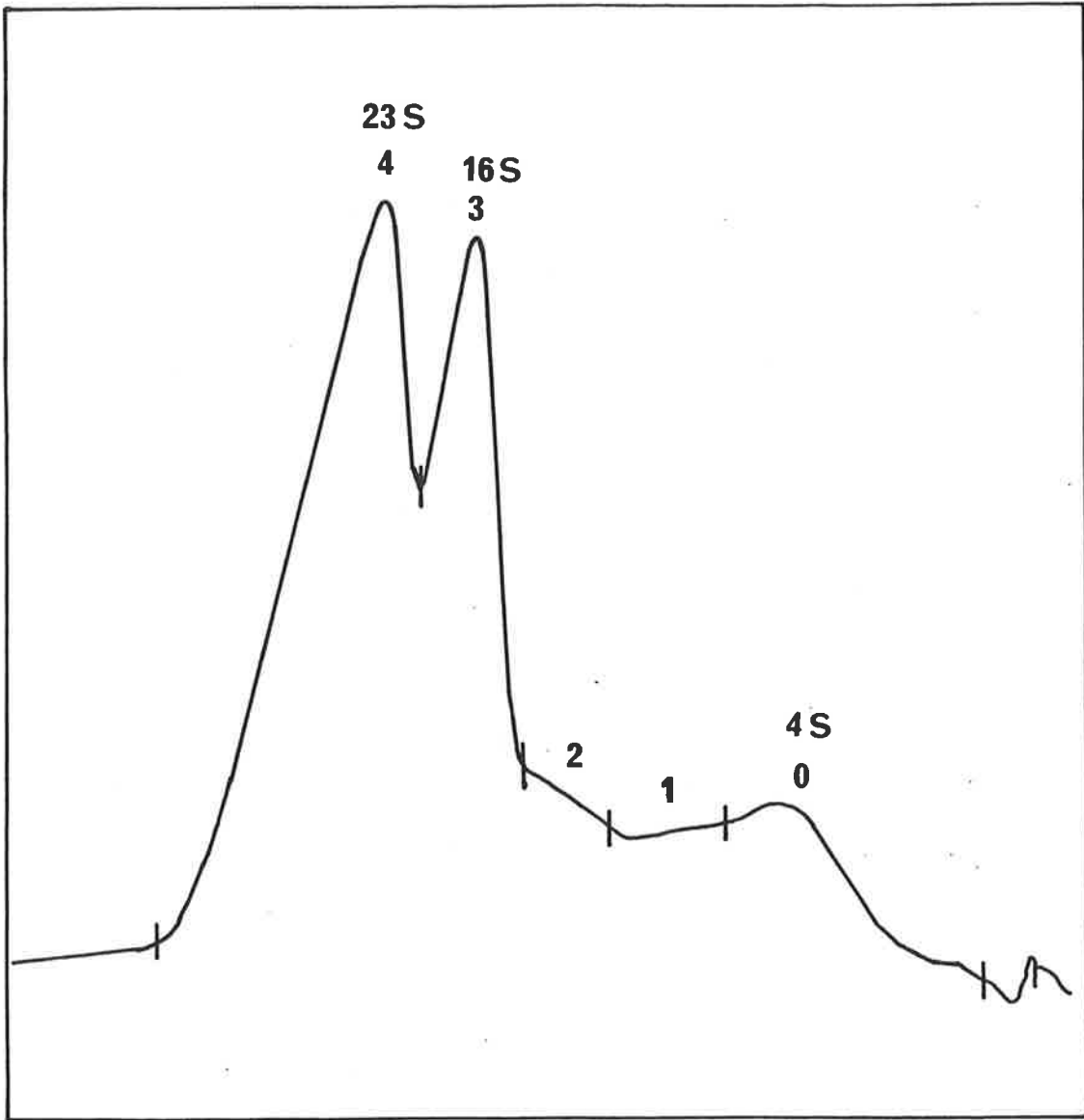
FIGURE 3.9

FORMAMIDE-SUCROSE GRADIENT OPTICAL DENSITY

PROFILE OF *B. AMYLOLIQUEFACIENS* 75 HAA RNA

75 Haa RNA was prepared, 200  $\mu\text{g}$  centrifuged on a 5-25% sucrose density gradient containing 70% formamide for 22 h, 20°C at 37,000 rpm, and the gradient band fractionated as indicated. Details of all preparations and procedures are provided in Methods, Chapter 2. The optical density profile (280 nm) is shown.

Optical Density (280nm)



Methylmercury gel analysis was carried out to examine the effectiveness of the fractionation. As can be seen in the photograph of Figure 3.10, this gradient system allows distinct fractionation of the total RNA. Translation of each RNA fraction demonstrated that essentially all the mRNA for the products of interest is contained in Fraction 2 RNA. The RNA of this fraction (which is in the region of 13 S) is of a size consistent with the previous work of O'Connor (1978), and represents a considerable enrichment of the mRNA of interest as most of the rRNA has been removed.

It is of interest to compare the profiles of RNA fractionated in the different gradient systems. The additional peak seen between the 4 S RNA and 16 S RNA peaks in the non-denaturing gradients (Figure 3.9) is absent for the most part in the denaturing formamide gradient profile (Figure 3.10). Preliminary work indicated that this peak is also reduced by brief heat treatment of the RNA prior to loading, and also by including 0.5% (w/v) SDS in the non-denaturing gradients. The 16 S and 23S rRNA peaks are also of different size in the two gradient systems, the 23 S peak being reduced in the denaturing gradients.

This information tends to suggest that much of the RNA in the peak between 16 S and 4 S is due to aggregates of RNA, possibly rRNA breakdown products, which are largely removed by the use of denaturing conditions in the gradient fractionation. The importance of employing these denaturing conditions in the fractionation of the RNA is emphasised by the above findings.

It was of interest to see whether this enriched mRNA

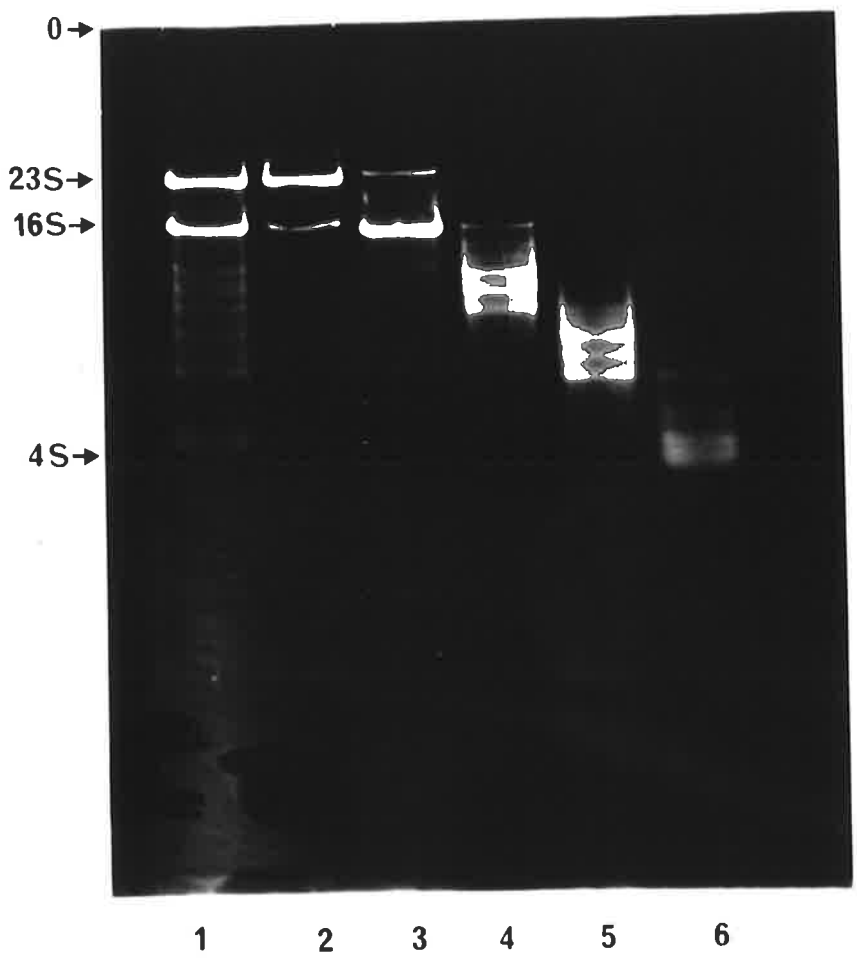
FIGURE 3.10

AGAROSE-METHYLMERCURY GEL ANALYSIS OF FORMAMIDE-  
SUCROSE GRADIENT FRACTIONS OF 75 HAA RNA

10  $\mu$ g of each of the formamide sucrose gradient RNA fractions obtained as described in Figure 3.9 were electrophoresed on a 1% agarose gel containing 5 mM methylmercury hydroxide as described in Methods, Chapter 2.

TRACKS

1. 10  $\mu$ g of 75 Haa total RNA (unfractionated)
2. Fraction 4 RNA
3. Fraction 3 RNA
4. Fraction 2 RNA
5. Fraction 1 RNA
6. Fraction 0 RNA



fraction could be further purified for the translation products of interest.

Finer fractionation of these denaturing gradients was attempted. However, recovery of RNA and translation was poor, as the size of the fractions became smaller. The accuracy of fractionation was not sufficient to allow pooling of small volumes from different gradients. The broad fractionation as shown in Figure 3.10 could be carried out reproducibly on a preparative scale. Further fractionation of the enriched mRNA obtained in this manner was attempted using preparative gel electrophoresis as described in the following section.

### 3.12 PREPARATIVE METHYLMERCURY GEL ELECTROPHORESIS OF THE ENRICHED mRNA FRACTION

Agarose-methylmercury gels had been used previously as an analytical tool for examining *B. amyloliquefaciens* RNA samples. It was decided to run the enriched mRNA fractionation obtained by formamide gradient (Fraction 2, Figures 3.9 and 3.10) preparatively on such a methylmercury gel, as described in Methods, Chapter 2, and by slicing the gel appropriately, further fractionate the RNA for recovery and translation.

In the first trials, 10  $\mu$ g of the enriched mRNA was loaded onto each of two tracks and six slices of about 2 mm thickness made down the gel starting from a 16 S RNA marker. Recovery of RNA from the gel slices was attempted using an electroelution procedure (Symons, 1978). However, this



method failed to yield significant amounts of translatable RNA as judged by assay in the *E. coli* cell-free system.

These trials were repeated, using low melting point agarose in the gels and extraction of RNA from the gel slices as described in Methods, Chapter 2. This method was found to give reproducible results and significant recovery of translatable RNA. Figure 3.11 shows a typical result in which a quarter of the RNA extracted from each gel slice was translated in the *E. coli* cell-free system. The mRNA's in the enriched mRNA preparation, (gradient Fraction 2 RNA, Track 1), are further separated out using this technique. In particular, slice 4 RNA contains almost all the mRNA for the translation products of interest, in the region of the culture supernatant alkaline protease of *B. amyloliquefaciens* (arrowed).

This partially purified mRNA of slice 4 is suitable for use in synthesis of a cDNA probe. This slice is well separated from the 16 S RNA on the gel and will therefore contain minimal rRNA. The further use of this partially purified mRNA fraction is described in the next Chapter.

### 3.13 DISCUSSION

The work described in this chapter has demonstrated by the criteria of immunoprecipitation and peptide mapping that translation products related to the extracellular alkaline protease are synthesised in the *E. coli* cell-free translation system in response to mRNA isolated from *B. amyloliquefaciens*.

During this work it became clear that the proposed pool of messenger RNA for protease could not be demonstrated by physical analysis of RNA isolated under conditions in which

### FIGURE 3.11

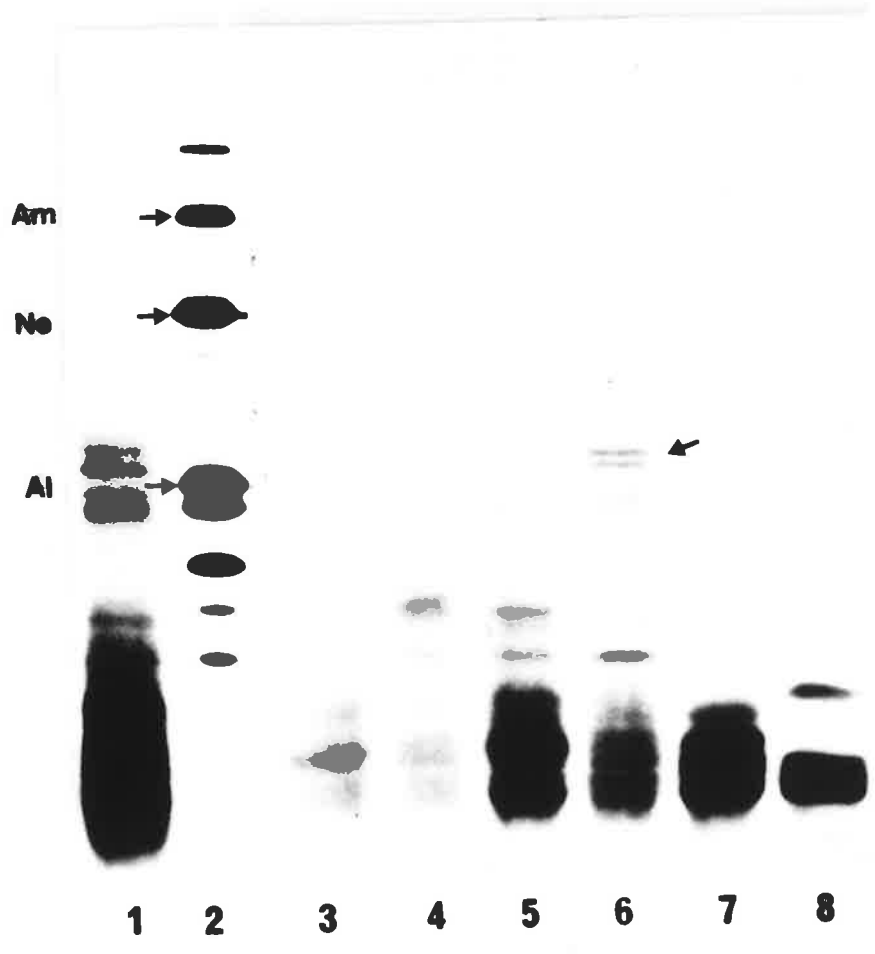
#### TRANSLATION PRODUCTS DIRECTED BY AGAROSE-METHYL- MERCURY GEL FRACTIONATED RNA IN THE *E. COLI* CELL- FREE SYSTEM

Gradient Fraction 2 RNA (10  $\mu$ g) obtained as described in Figure 3.9 was electrophoresed on each of two Tracks of a 1% low-melting-temperature-agarose gel containing 5 mM methylmercury hydroxide as described in Methods, Chapter 2. Using 75 Haa RNA (10  $\mu$ g) in adjacent Tracks to provide rRNA markers, 6 gel slices of approximately 2 mm in width were excised from the gel, (downwards 1-6 from the 16 S rRNA marker) across the Tracks containing the Fraction 2 RNA. The RNA was extracted from the gel slices as described in Methods, Chapter 2, and dissolved finally in 20  $\mu$ l of water. 5  $\mu$ l ( $\frac{1}{2}$  of each sample) was translated in the *E. coli* cell-free system. Gradient RNA Fraction 2 (10  $\mu$ g) was also translated, and the samples electrophoresed on a 12.5% polyacrylamide gel which was processed by fluorography and autoradiographed. Details of all preparations and procedures are provided in Methods, Chapter 2. [ $^{35}$ S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens* were used as markers and the major enzymes are labelled.

#### TRACKS of translation products directed by:

1. Gradient Fraction 2 RNA
2. [ $^{35}$ S]-methionine labelled culture supernatant protein of *B. amyloliquefaciens*
3. Slice 1 RNA
4. Slice 2 RNA
5. Slice 3 RNA
6. Slice 4 RNA
7. Slice 5 RNA
8. Slice 6 RNA

Am  $\equiv$   $\alpha$ -amylase  
Ne  $\equiv$  neutral protease  
Al  $\equiv$  alkaline protease



the pool is proposed to be large or small. The proposed pool is also not readily demonstrable by qualitative or quantitative differences in the translation products of RNA isolated under "high" or "low" proposed pool conditions.

A translation product of slightly slower mobility (in the SDS gel system) than native alkaline protease was shown by immunoprecipitation and peptide mapping to be a likely candidate for a precursor form of this protein. As detailed in Section 3.10, a somewhat puzzling and disconcerting result is the relative decrease of this species in immunoprecipitates of total translation products directed by messenger RNA isolated from cells incubated in low amino acids medium (i.e., 60 Laa RNA) when compared to the immunoprecipitates from the products directed by messenger RNA isolated from cells incubated in high amino acids medium only (i.e., 75 Haa). This seems to be the reverse of the situation we would predict on the basis of our earlier work. That is, RNA isolated from cells incubated in high amino acids as described in Section 3.5 would be expected to contain less mRNA coding for protease than RNA isolated from cells optimized for the "pool" by incubation in low amino acids medium.

Work to be described in later chapters indicated that the mRNA for alkaline protease is indeed more abundant in the 60 Laa RNA preparation. However, this was not evident from the experiments presented here. At this stage, as discussed in Section 3.10, in this *in vitro* work, we cannot assume that our pool hypothesis is correct, and therefore, further fractionation of the mRNA was conducted, using as starting material RNA readily demonstrable to contain mRNA coding for translation products related to the alkaline protease.

Fractionation of this RNA by gradient analysis and preparative gel electrophoresis resulted in isolation of a fraction greatly enriched for the translation products of interest. The following chapter describes the use of this partially purified mRNA in synthesis of a cDNA probe for use in screening a genomal library of *B. amyloliquefaciens* DNA.

CHAPTER FOUR

ISOLATION FROM A GENOMAL LIBRARY OF *B. AMYLOLIQUEFACIENS* DNA  
OF A PUTATIVE ALKALINE PROTEASE CLONE

#### 4.1 INTRODUCTION

The previous Chapter dealt with the isolation of mRNA from *B. amyloliquefaciens* and translation of this message in a cell-free system derived from *E. coli*. Using the techniques of immunoprecipitation and peptide mapping translation products have been identified, which, by these criteria are related to the alkaline protease of *B. amyloliquefaciens*.

Fractionation of the RNA of *B. amyloliquefaciens* as described in Chapter 3, resulted in partial purification of the messenger RNA for a related translation product which has a molecular weight a little larger than the culture supernatant alkaline protease. As mentioned previously, the main aim of this work was to provide a probe suitable for use in isolation of the gene for the alkaline protease of *B. amyloliquefaciens*.

Construction of cDNA clones using the enriched messenger RNA as a starting material was considered as a cloning strategy. This approach is often used in isolating genes from eukaryotic organisms which in general, have genomes many times larger than a prokaryote genome. Given the comparatively small size of the bacterial genome (a single chromosome, which in the case of *B. amyloliquefaciens* is approximately 4500 Kb in size), construction of a genomal library is a relatively simple undertaking. It was therefore decided to set up a genomal library from *B. amyloliquefaciens* chromosomal DNA and to screen it directly using as a probe, cDNA synthesised from the partially purified messenger RNA fraction. This work is presented in this chapter.

## 4.2 CONSTRUCTION OF A GENOMAL LIBRARY OF

### *B. AMYLOLIQUEFACIENS* DNA

Colony libraries have been constructed using plasmid vectors in *E. coli* for a number of prokaryote organisms. These include colony banks for *B. subtilis* 168 (Rapoport *et al.* 1979), *Beneckea harveyi* (Lamfrom *et al.*, 1978) and *E. coli* (Clarke & Carbon, 1976). The most commonly used procedures for preparing genomal DNA for cloning are random shearing and use of the poly(dA·dT) "connector" method of Lobban & Kaiser, (1973); or partial restriction digestion of the DNA prior to ligation into the plasmid vector of choice. Starting with genomal DNA of high molecular weight, the aim of these procedures is to obtain DNA fragments randomly representative of the genome, of a size suitable for cloning into the vector system chosen.

Construction of a colony library of *B. amyloliquefaciens* DNA in *E. coli* using the plasmid vector pBR322 was chosen as this system was well characterised and readily available. Using the probability calculation of Clarke & Carbon (1976), assuming the fragments cloned are 10 Kb in length and the desired gene approximately 1 Kb, it was estimated that in a colony library of 1500 clones, the probability of finding the desired gene intact is 95%.

The following work describes the construction of a colony library of about 2000 clones for initial screening using the enriched mRNA, prepared as described in Chapter 3, as a probe.



### 4.3 ISOLATION OF CHROMOSOMAL DNA FROM

#### *B. AMYLOLIQUEFACIENS*

Chromosomal DNA of high molecular weight is required, as mentioned, for the construction of a colony library representative of the *B. amyloliquefaciens* genome. To provide this material, chromosomal DNA was isolated from 240 mls of an LF<sup>-</sup> strain culture of *B. amyloliquefaciens* as described in Methods, Chapter 2. Protoplasts were formed initially to facilitate gentle disruption of the cells during protease K treatment.

A sample of the DNA isolated by this procedure was analysed on a 1% agarose gel (as in Methods, Chapter 2) using as markers *Hind*III digested  $\lambda$  DNA, and was shown to be in excess of 30 kilobases in length.

### 4.4 PARTIAL DIGESTION AND SIZE

#### FRACTIONATION OF THE DNA

DNA of about 10 kilobases in length was considered a suitable size for construction of a colony library in *E. coli* in the plasmid vector pBR322. To construct a library with as near as possible a random collection of clones representative of the entire genome, cloning of randomly sheared DNA fragments is the procedure of choice.

Attempts to shear the preparation of *B. amyloliquefaciens* DNA to a size suitable for cloning in pBR322, using standard procedures were unsuccessful.

An alternative method for preparing DNA of a suitable size for cloning is the use of controlled partial digestion

with a restriction endonuclease known to cleave the DNA frequently. The restriction enzymes with "4-base" recognition sequences are useful for this purpose. An example of the use of this approach was the construction of a library of *B. amyloliquefaciens* DNA in *B. subtilis* (Palva, 1982), which enabled isolation of the *B. amyloliquefaciens*  $\alpha$ -amylase gene as was discussed in Chapter 1.

Random fragments of DNA are generated by partial cleavage of the starting material using various amounts of enzyme and various times of incubation, and the size of fragment required for cloning selected.

As a rough approximation, a restriction enzyme with a "4-base" recognition sequence will cleave DNA on an average of every 256 bases. By selecting cleaved material 10 Kb in length, approximately one in 40 sites on average have been cleaved.

An advantage of this approach is that secondary modifications to the DNA prior to ligation into the plasmid vector (such as the addition of linkers or tails) required when sheared DNA is used, can be avoided.

In preliminary trials, the DNA of *B. amyloliquefaciens* was digested with a range of different "4-base" restriction enzymes, using various amounts of enzyme in the reaction mixes and for various incubation times. The standard conditions for restriction digests are given in Methods, Chapter 2.

The restriction enzyme *Sau3A* proved to be the most reliable and amenable to controlled digestion. It is also convenient in that DNA fragments with *Sau3A* cohesive ends can be directly ligated into the *Bam*HI site of the plasmid

vector pBR322.

To generate DNA fragments for cloning, cleaved as far as possible in a random manner, four different preparative *Sau*3A digestions were carried out, using the conditions determined in the analytical trials conducted previously. These are described in Table 4.1. The partially digested DNA was electrophoresed on a 1% low melting point agarose gel as described in Methods, Chapter 2, briefly stained with ethidium bromide to visualise the *Hind*III digested  $\lambda$  DNA markers, and the DNA of molecular weight approximately 8 - 12 Kb cut out and isolated from the low melting point agarose as described in Methods, Chapter 2.

#### 4.5 CONSTRUCTION OF THE GENOMAL LIBRARY OF *B. AMYLOLIQUEFACIENS* DNA

Samples of the 8 - 12 Kb, partially digested DNA were ligated into *Bam*HI cleaved plasmid pBR322. The details of the ligation reaction and preparation of the vector are given in Methods, Chapter 2. Insertion of a DNA fragment into the *Bam*HI site of pBR322 results in insertional-inactivation of the tetracycline resistance gene carried on this plasmid. The ligated material was used to transform competent cells of *E. coli* strain MC1061 as described in Methods, Chapter 2, and the transformants selected by plating on Luria broth agar plates containing 50  $\mu$ g/ml ampicillin.

A colony library in excess of 2000 clones was obtained, (the platings of transformants being done at a colony density which allowed direct selection of individual clones). Each colony was picked and placed in an order array on fresh plates

TABLE 4.1

PARTIAL SAU3A RESTRICTION DIGESTION AND SIZE  
FRACTIONATION OF B. AMYLOLIQUEFACIENS DNA

Digest	1	2.	3	4
$\mu$ g <i>B. amyloliquefaciens</i> DNA	20	20	20	20
<i>Sau</i> 3A (units)	1	1	1	2
incubation time (min)	2	5	10	2

Four 30  $\mu$ l reactions composed as above using conditions for the restriction endonuclease *Sau*3A as described in Methods, Chapter 2 where incubated at 30°C for the times indicated. The digests were electrophoresed on a 1% low melting temperature agarose gel and using *Hind*III digested  $\lambda$  DNA as markers, the region of the gel corresponding to digested *B. amyloliquefaciens* DNA of approximately 8-12 Kb was excised and the DNA isolated from the low melting temperature agarose. (Details of all preparations and procedures are provided in Methods, Chapter 2). (This DNA was used as described in Section 4.5 in construction of a genomal library).

containing ampicillin or tetracycline. The vector had been treated with calf intestinal phosphatase to reduce self-ligation, and over 80% of the ampicillin resistant transformants were shown to be tetracycline sensitive. Therefore these clones, approximately 2000 in all, carry a fragment of *B. amyloliquefaciens* DNA within the plasmid pBR322 (i.e. recombinant plasmids). To check the insert size of these clones, minipreparations of plasmid DNA from twenty colonies selected at random were carried out. Restriction analysis showed that all plasmids carried inserts of at least 8 Kb.

It had been estimated previously that a library of about 1500 clones, each with an insert size of approximately 10 Kb would be sufficient to cover the genome of *B. amyloliquefaciens*, with a high probability of containing a given region of 1 Kb in length.

The library of about 2000 clones constructed above was used in screening for the alkaline protease gene of *B. amyloliquefaciens*. Glycerol stocks of all clones were prepared and stored in 96 well trays as described in Methods, Chapter 2.

#### 4.6 SCREENING OF THE *B. AMYLOLIQUEFACIENS* DNA LIBRARY

##### WITH A RANDOM-PRIMED cDNA PROBE

The library of approximately 2000 clones was re-picked from the ordered master plates and the colonies grown on circular nitrocellulose filters placed on fresh ampicillin plates. The colony filters were prepared for screening using the procedure of Grunstein & Hogness (1975), with

modifications as described in Methods, Chapter 2.

The hybridisation probe used for screening was random primed cDNA prepared as described in Methods, Chapter 2, using the partially purified messenger RNA, fraction 4, prepared as described in Section 3.11 of Chapter 3. This RNA material was confirmed by translation analysis to contain the translation products of interest (see Figure 3.11).

Figure 4.1 shows an autoradiogram of a selection of the nitrocellulose filters and gives the details of the hybridisation and washing conditions used. Each filter represents 96 individual clones in duplicate in an ordered array. Colonies showing hybridisation to the probe with varying degrees of intensity can be seen in the autoradiogram as indicated.

From all of the filters screened (i.e. the complete library) 28 positives of varying strength of hybridisation signal were obtained. These were picked onto a fresh master plate for further screening and glycerol stocks prepared for storage as described in Methods, Chapter 2.

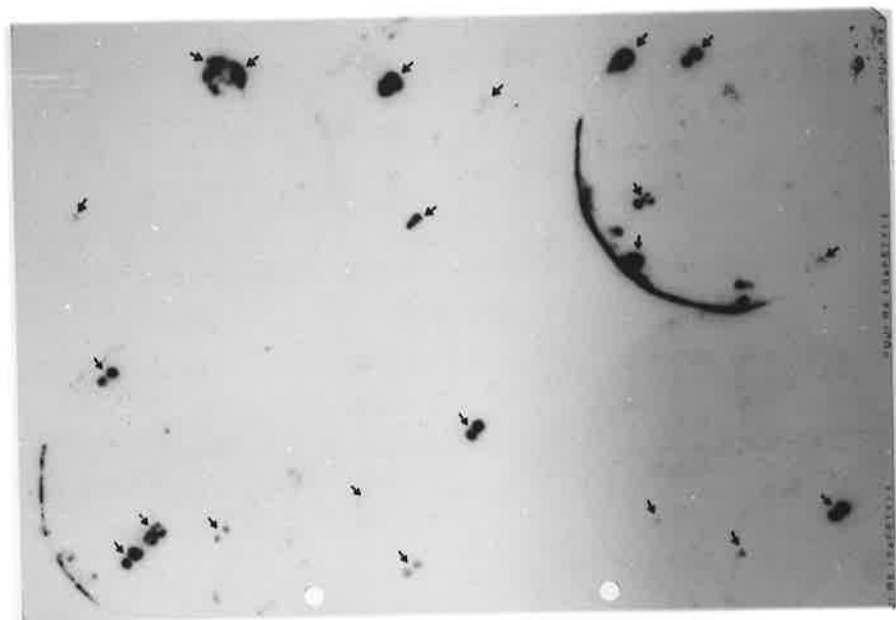
#### 4.7 NEGATIVE SCREENING OF POSITIVE RECOMBINANTS WITH RIBOSOMAL RNA PROBES

The twenty-eight positive recombinants obtained were re-screened as described in Section 4.6 using this time as a probe random primed cDNA made from the ribosomal RNA's of *B. amyloliquefaciens*. The mRNA preparation used in the initial screening is likely to contain an amount of broken down rRNA which will contribute in the cDNA probe synthesis. Therefore to reduce the number of positives to be screened

FIGURE 4.1

SCREENING OF THE COLONY LIBRARY OF *B. AMYLOLIQUEFACIENS* DNA WITH THE RANDOM-PRIMED cDNA PROBE

Nitrocellulose filters were prepared from the colony library of *B. amyloliquefaciens* DNA constructed as described in Section 4.5 and Methods, Chapter 2. The library was screened by hybridisation with random-primed cDNA synthesised using partially purified mRNA Fraction 4 of *B. amyloliquefaciens* as described in Chapter 3, Section 3.11. Preparation of the random-primed cDNA and the nitrocellulose filters and the hybridisation and washing conditions used to screen the filters are given in Methods, Chapter 2. After washing, the filters were air dried and autoradiographed for 2 days at -80°C. Shown are the results for six filters, each comprising 96 individual clones in duplicate. The positive clones selected are indicated.





initially in the next stage, those clones hybridising with ribosomal RNA can be set aside for the time being.

The ribosomal cDNA probe was prepared, as follows. The two species of ribosomal RNA (16 S and 23 S) were purified as single bands from a preparative low melting point agarose - methylmercury gel of total *B. amyloliquefaciens* RNA. Random primed cDNA was made from each rRNA species in two separate reactions and equal amounts of  $^{32}\text{P}$ -cDNA from each reaction combined to form the ribosomal probe. The details of the above procedures are provided in Methods, Chapter 2.

Figure 4.2 shows the result of the screening of the 28 positives with the ribosomal probe. All but nine hybridise strongly with cDNA made to purified rRNA.

#### 4.8 CHARACTERISATION OF POSITIVE RECOMBINANT CLONES BY HYBRID-SELECTION TRANSLATION

A means for identifying clones related to particular messenger RNA's is provided by the technique of hybrid selection translation. In this procedure, the cloned DNA of interest is used to "select" by hybridisation, the mRNA with which it corresponds. The mRNA can then be recovered and characterised, by translation assay. Examples of the use of this technique in the isolation of specific mRNA's are provided by Ricciardi *et al.* (1981) and Bozzoni *et al.* (1981).

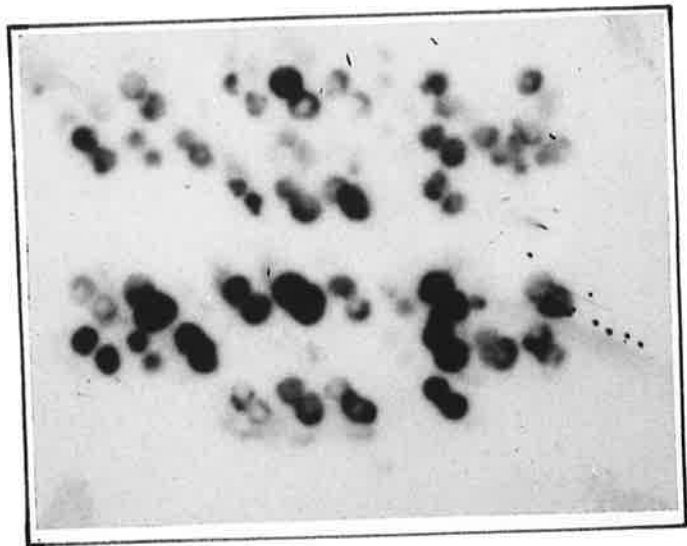
A procedure based on these examples was developed for the further characterisation of the positive clones obtained in Section 4.7, the details of which are provided in Methods, Chapter 2. Initially, the nine non-ribosomal clones obtained were chosen for immediate screening using this hybrid

FIGURE 4.2

SCREENING OF THE 28 POSITIVES WITH THE RIBOSOMAL

RNA PROBE

A nitrocellulose filter of the 28 positives obtained from the first round screening of the colony library as described in Section 4.6, was prepared as described in Figure 4.1 and Methods, Chapter 2. The filter was hybridised with the rRNA probe prepared as described in Methods, Chapter 2, as detailed in Figure 4.1, and autoradiographed.



selection translation technique. The hybrid selection procedure requires the use of 20 - 40  $\mu$ g of plasmid DNA from each clone. However, two of the nine clones would not grow in liquid culture, suggesting some inherent instability in these clones. Several lower amounts of ampicillin were tried than that used in the standard plasmid preparation method, and even growth in the absence of the antibiotic, with no success. Therefore it was decided to proceed with plasmid DNA preparations of the remaining seven clones (designated 1  $\rightarrow$  7).

Plasmid DNA from these seven clones was bound to small nitrocellulose millipore filters and hybridised with total RNA of *B. amyloliquefaciens*. After washing to remove RNA not tightly bound, the remaining RNA still bound to the filters was eluted and prepared for translation. The details are given in Methods, Chapter 2.

Each RNA sample was translated in the *E. coli* cell-free system along with the appropriate controls. Figure 4.3 shows the result of the experiment when RNA isolated from *B. amyloliquefaciens* cells incubated for seventy-five minutes in high amino acids medium (i.e. 75 Haa RNA) was used. The result was qualitatively the same when 60 Laa RNA was used. The characteristics of these RNA preparations were discussed in detail in Chapter 3.

The autoradiogram of the hybrid selection-translation trial presented in Figure 4.3 shows that clones selecting three of the four major translation products in the region of the alkaline protease have been obtained as indicated. Of particular interest is Clone 5, which strongly selects a translation product which comigrates on SDS gels with a translation product shown in Chapter 3 to be specifically

FIGURE 4.3

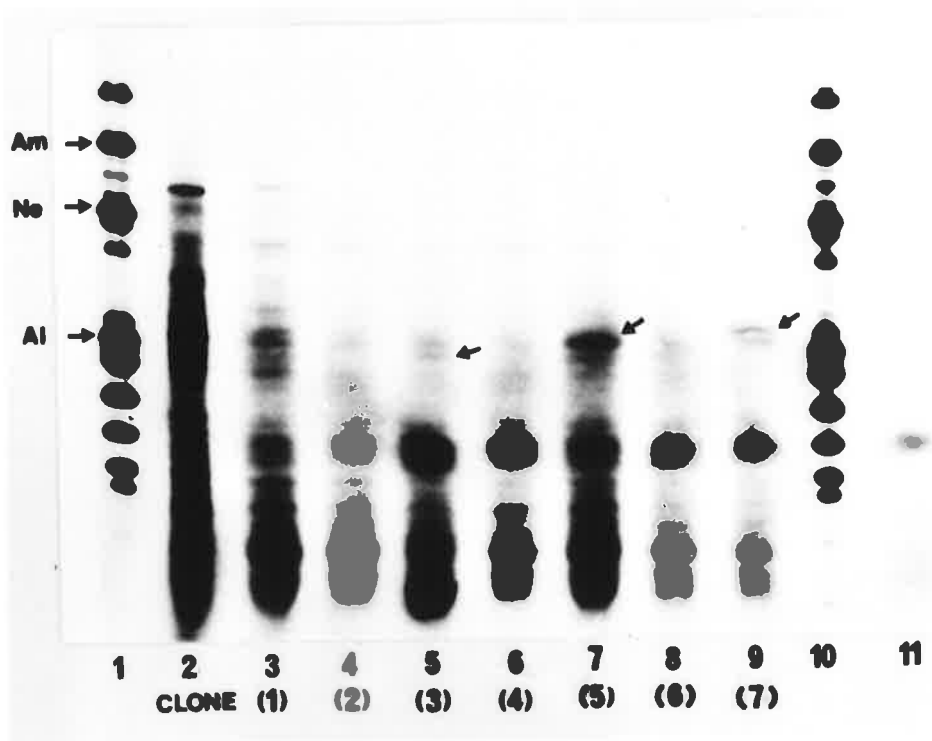
HYBRID SELECTION TRANSLATION ANALYSIS OF  
RECOMBINANTS: TRANSLATION PRODUCTS DIRECTED  
BY HYBRID SELECTED mRNA FROM POSITIVE CLONES  
1-7, IN THE *E. COLI* CELL-FREE SYSTEM

Nitrocellulose filters were prepared, each with 40 µg of plasmid DNA from clones 1-7 and hybridised with 200 µg of 75 Haa RNA (prepared as described in Chapter 3) using the hybrid-selection technique detailed in Methods, Chapter 2. The hybrid selected RNA was eluted from the filters and translated in the *E. coli* cell-free system. 75 Haa RNA was also translated. Samples of the translation products were electrophoresed on a 12.5% polyacrylamide gel (also with [<sup>35</sup>S]-methionine labelled culture supernatant proteins of *B. amyloliquefaciens* as markers) and the gel autoradiographed after processing by fluorography. Details of all preparations and procedures are provided in Methods, Chapter 2.

TRACKS

1. [<sup>35</sup>S]-methionine labelled *B. amyloliquefaciens* culture supernatant proteins
2. 75 Haa RNA total translation products
3. Clone 1 hybrid selected mRNA products
4. Clone 2 hybrid selected mRNA products
5. Clone 3 hybrid selected mRNA products
6. Clone 4 hybrid selected mRNA products
7. Clone 5 hybrid selected mRNA products
8. Clone 6 hybrid selected mRNA products
9. Clone 7 hybrid selected mRNA products
10. [<sup>35</sup>S]-methionine labelled *B. amyloliquefaciens* culture supernatant proteins
11. No added RNA (control products)

Am ≡ α-amylase  
Ne ≡ neutral protease  
Al ≡ alkaline protease



immunoprecipitated with the antisera to the culture supernatant alkaline protease. As was mentioned, the result was the same when 60 Laa RNA was used in a repeat hybrid selection experiment, although the immunoprecipitation of the corresponding translation product was shown in Chapter 3 to be relatively weak when compared to that of the 75 Haa RNA translation product. A possible reason for this is discussed in Chapter 3.

We therefore have a clone which selects from 75 Haa RNA and 60 Laa RNA total translation products, the same product (by molecular weight) which is immunoprecipitated using antisera to the alkaline protease as described in Chapter 3.

To check that this selected product is immunoprecipitated, the hybrid-selection-translation experiment was repeated using Clone 5 plasmid DNA and a sample of the translation products immunoprecipitated. Although the result was very faint, due to the small amounts of translated material resulting from hybrid selection followed by immunoprecipitation, the antisera specifically immunoprecipitated this product. This result is not presented here as it was very poorly reproducible, but nonetheless, indicated that further characterisation of this clone by DNA sequence analysis was required. That is, the positive conclusion from these hybrid-selection-translation and immunoprecipitation experiments is that the clone in question selects for a translation product which is related by immunoprecipitation to the alkaline protease of *B. amyloliquefaciens*. Therefore, further characterisation of this clone (Clone 5) by DNA sequence analysis, was warranted.

#### 4.9 RESTRICTION MAPPING AND SOUTHERN ANALYSIS OF CLONE 5 PLASMID DNA

Preparatory to DNA sequencing, plasmid DNA of Clone 5 was digested with a range of restriction enzymes, which were probed by Southern analysis as described in Methods, Chapter 2, using the cDNA probe employed previously to screen the colony library.

The results of these experiments, showed that in the *Hind*III digest, only two small fragments (0.7 Kb and 1.7 Kb in size) hybridised with the cDNA probe. Restriction mapping showed these two fragments to be linked.

#### 4.10 DNA SEQUENCE ANALYSIS OF THE 1.7 Kb AND 0.7 Kb *HIND*III FRAGMENTS

These *Hind*III restriction fragments were extensively sequenced in both directions from the internal *Hind*III restriction site, using the M13 dideoxy sequencing technique detailed in Methods, Chapter 2. As a starting point, the 1.7 Kb and 0.7 Kb fragments were isolated, cloned into M13 and sequenced from each end. *Sau*3A and *Hpa*II digests of the *Hind*III pieces were then carried out and these fragments isolated, cloned into M13 and sequenced.

The DNA sequence data obtained was analysed for protein coding sequences in all six possible reading frames. Several regions containing open readings frames were disclosed, however, none showed amino acid sequence homology with the published sequence of the alkaline protease of *B. amyloliquefaciens*.

This was a disappointing and somewhat puzzling result.



However, at this stage of the work, an alternative approach became available, as will be described in detail in the following chapter, for the isolation of the alkaline protease gene; that of the use of synthetic oligonucleotide DNA sequences as hybridisation probes.

The preliminary work to be described in the next chapter, using these synthetic probes, showed great promise of achieving isolation of the specific gene desired.

#### 4.11 RESCREENING OF THE POSITIVE RECOMBINANT CLONES WITH THE NEW SYNTHETIC PROBES

Prior to the use of these new probes (details of which are given in the next chapter) in the screening of a new colony library, plasmid DNA of the 26 viable recombinant clones obtained through the present work were tested by dot hybridisation for homology with the synthetic probes. It was thought possible, for example that if the desired gene was linked to genes for ribosomal RNA's, that a positive may have been eliminated by the ribosomal screening carried out in Section 4.7. It is also possible that the hybrid-selection results were misleading. However, none of the 26 positives showed hybridisation with the new synthetic probes.

To check the hybrid-selection positive clone (Clone 5) obtained in Section 4.8 more rigourously, restriction fragments of this putative protease clone were screened by Southern analysis with the synthetic probes. Again the result was negative. The possibility exists that this clone is made up of DNA not contiguous in the *B. amyloliquefaciens* chromosome; for example this could arise through ligation of

two or more *Sau*3A fragments into a single pBR322 vector molecule. While this possibility seems remote, if it were the case, then this clone may contain DNA specific for the protease mRNA, but not containing protein coding sequence.

The contiguity of the DNA in this clone could be checked by hybridisation back to the appropriate restriction digest of *B. amyloliquefaciens* genomic DNA.

It is also possible that the library constructed here was not representative of the entire genome of *B. amyloliquefaciens*, although measures were taken to try and ensure cloning of random fragments. Hence the gene of interest may have been missed. This possibility also seems unlikely.

However in view of the above, a potentially more fruitful and more direct approach, in line with the overall aims of this work, (that is to isolate the alkaline protease gene), was to pursue the use of the synthetic DNA hybridisation probes, in the screening of a new genomic library of *B. amyloliquefaciens* DNA. This work is described in the following chapter.

#### 4.12 DISCUSSION

The work presented in this chapter described the use of a partially purified mRNA preparation in the screening of a genomic colony library of *B. amyloliquefaciens* DNA, with the aim of isolating the gene for the extracellular alkaline protease of this organism.

As a result of this work, a clone was isolated which by the criteria of hybrid-selection-translation appeared to be related to the gene of interest. However, DNA sequencing

of this clone did not confirm this.

This result was disappointing and indicated that a new approach for the isolation of the alkaline protease gene of *B. amyloliquefaciens* would be required. The putative alkaline protease clone isolated in this work was shown using the hybrid-selection technique to select mRNA coding for a translation product, just larger than the culture supernatant alkaline protease, which was immunoprecipitated with antiserum prepared to the culture supernatant enzyme.

This antiserum was shown in Chapter 3 (Section 3.7) to react weakly with the culture supernatant neutral protease. A possible explanation for the above work therefore, is that the hybrid selected translation product is related (as a breakdown product) to the neutral protease of *B. amyloliquefaciens*. This possibility will be investigated when the amino acid sequence of this enzyme becomes available.

During the sequencing work described in this chapter it became possible for me to obtain synthetic probes, one commercially and one synthesised in this laboratory, corresponding to different regions of the alkaline protease peptide chain. As the DNA sequencing results continued to fail to produce any expected coding sequence, the attraction of switching the attack to the use of synthetic probes became more pressing.

As described in the next chapter, this approach was successfully adopted. The clone isolated in the present chapter may well be of some interest, but it was decided to press on with the new approach and perhaps look at this clone at some later date.

CHAPTER FIVE

ISOLATION OF THE GENE FOR THE EXTRACELLULAR  
ALKALINE PROTEASE OF *B. AMYLOLIQUEFACIENS*

## 5.1 INTRODUCTION

The use of synthetic DNA oligomers as hybridisation probes has become a powerful tool in the isolation of specific genes. The work of Wallace *et al.* (1981) has provided a set of conditions which, with simple modifications, has allowed their use in the isolation and cloning of genes from a wide variety of sources. They are short sequences of synthetic DNA (generally 11 - 20 nucleotides in length) which can be used, after end-labelling as direct hybridisation probes (for examples see Suggs *et al.* [1981] and Stetler *et al.* [1982]) or as primers for the synthesis from messenger RNA of extended cDNA copies for use as probes or as the starting material for cDNA cloning, (Chang *et al.*, 1981). This technology has been particularly useful in the isolation of cDNA clones corresponding to low abundance messenger RNA's.

Therefore, if the protein sequence (or part thereof) of a particular gene product is known, a segment of this sequence can be used to design a synthetic oligomer for use in isolation of a specific gene.

This chapter describes the use of this approach in the isolation of the gene for the extracellular alkaline protease of *B. amyloliquefaciens*, and the partial characterisation of this gene.

## 5.2 DESIGN OF SYNTHETIC OLIGONUCLEOTIDES CORRESPONDING TO THE ALKALINE PROTEASE GENE SEQUENCE

The extracellular alkaline protease of *B. amyloliquefaciens* (also classified as subtilisin BPN') has been fully sequenced

(Markland & Smith, 1967). It is two hundred and seventy-five amino acids long and has a molecular weight of 27,534. The alkaline protease from the strain of *B. amyloliquefaciens* used in this laboratory has been purified to homogeneity (Love, 1981; O'Connor, personal communication). Protein sequencing of the first forty amino acids of this enzyme showed identity with the published sequence (O'Connor, personal communication), and therefore the entire sequence can reasonably be used in the selection and design of oligonucleotide probes. The choice of two fourteen-mer sequences was made after examination of the amino acid sequence of the protein. Figure 5.1 shows the two mixtures of sequences selected, along with the region of protein sequence from which they were derived. These two stretches of five amino acids were chosen because they involve use of the least possible number of degeneracies in the amino acid codons.

They are designated as 14-mer 1 and 14-mer 2 and consist of mixtures of eight and sixteen oligonucleotides respectively.

These synthetic oligonucleotide mixtures were used as hybridisation probes (as described below) in the screening of a genomal library of *B. amyloliquefaciens* DNA. The rationale for the use of two stretches of sequence from different regions of the protein molecule was to increase the probability of specific hybridisation with genomal DNA. That is, when screening a genomal library, depending on the stringency of washing conditions used, non specific hybridisation of these relatively short oligonucleotide sequences may occur. The probability of this non-specific hybridisation

FIGURE 5.1

SYNTHETIC OLIGONUCLEOTIDES SPECIFIC FOR THE  
ALKALINE PROTEASE GENE OF *B. AMYLOLIQUEFACIENS*

14-mer 1	Amino acid No.	117	118	119	120	121
	N-t...	ASN	ASN	MET	ASP	VAL ...C-t
	mRNA 5'...	AA <sup>U</sup> <sub>C</sub>	AA <sup>U</sup> <sub>C</sub>	AUG	GA <sup>U</sup> <sub>C</sub>	GU ...3'
	cDNA 3'... (primer)	TT <sup>A</sup> <sub>G</sub>	TT <sup>A</sup> <sub>G</sub>	TAC	CT <sup>A</sup> <sub>G</sub>	CA ...5'

+ a mixture of 8 x 14-mer sequences

14-mer 2	Amino acid No.	238	239	240	241	242
	N-t...	HIS	PRO	ASN	TRP	THR ...C-t
	mRNA 5'...	CA <sup>U</sup> <sub>C</sub>	CC <sup>C</sup> <sub>A</sub> G	AA <sup>U</sup> <sub>C</sub>	UGG	AC ...3'
	cDNA 3'... (primer)	GTA G	GGA G T C	TTA G	ACC	TG ...5'

- a mixture of 16 x 14-mer sequences

occurring with both probes to the same fragment of cloned genomic DNA is small and therefore, clones which show positive hybridisation to both probes are highly likely to be specific for the probes used. (This assumes that the restriction enzyme chosen when constructing a genomal library does not result in cleavage of the DNA such that the probes hybridise to different restriction fragments. As will be seen in the work to follow this has not been a problem).

A final point about the design of the fourteen-mers is that they were chosen to be complementary to the messenger RNA strand to enable their use in primer extension work should this be necessary (this was found to be the case - see Section 5.6).

### 5.3 SEQUENCE ANALYSIS OF THE SYNTHETIC FOURTEEN-MERS

The two fourteen-mers described in Section 5.2 were obtained from different sources as detailed in Materials, Chapter 2. Prior to use as hybridisation probes they were sequenced using the chemical cleavage techniques of Maxam & Gilbert (1980) as modified by Banaszuk *et al.* (1983) for sequencing of oligodeoxyribonucleotides and were confirmed to be as shown in Figure 5.1.

### 5.4 SOUTHERN ANALYSIS OF *B. AMYLOLIQUEFACIENS*

#### DNA USING 14-MERS 1 AND 2 AS HYBRIDISATION PROBES

To test the fourteen-mers for specificity when hybridised to genomic DNA of *B. amyloliquefaciens*, Southern blot analysis of the DNA digested with *Hind*III, *Eco*RI or *Bgl* II was carried out



as described in Methods, Chapter 2, using the [ $^{32}\text{P}$ ]-phosphorylated 14-mer mixtures 1 or 2 as probes. The hybridisation signals from these trials, using various amounts of digested DNA were faint and the data is not presented here as the results were not photo-reproducible.

However, visual inspection of autoradiograms showed that in each of the three digests, both 14-mer probes hybridised to single restriction fragments of the same molecular weight. The fragment hybridised by both 14-mers in the *Hind*III digests was the smallest, being approximately 2.6 Kb in size (as determined using *Hind*III digested  $\lambda$  DNA markers). This fragment was chosen for cloning.

## 5.5 SIZE SELECTION OF *HIND*III DIGESTED

### *B. AMYLOLIQUEFACIENS* DNA FOR CLONING

To prepare material for construction of a new library of *B. amyloliquefaciens* DNA fragments, the DNA was digested with *Hind*III on a preparative scale and electrophoresed on a 1% low melting-point agarose gel as described in Figure 5.2. The *Hind*III fragment of interest lies between the 2.26 Kb - 4.26 Kb  $\lambda$  markers and therefore after staining to visualise the DNA this region was cut into 4 sections of equal width and the DNA isolated.

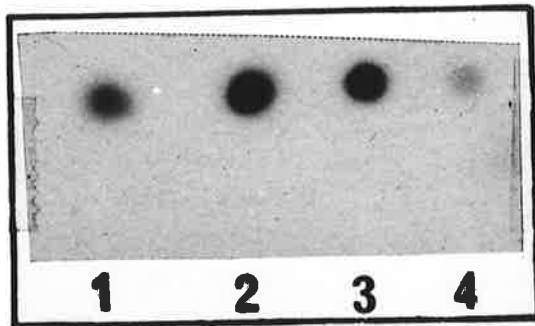
Dot hybridisation of these DNA fractions presented in Figure 5.2 using 14-mer 1 as probe showed that samples 2 and 3 contained about the same amount of hybridising material, indicating perhaps some spreading or overloading of the preparative gel.

FIGURE 5.2

SIZE SELECTION AND DOT BLOT ANALYSIS OF HINDIII

DIGESTED DNA OF *B. AMYLOLIQUEFACIENS*

DNA of *B. amyloliquefaciens* (120 µg) was digested with *Hind*III in 6 x 40 µl reactions each containing a 2-fold excess of enzyme for 4 h at 37°C. The digests were electrophoresed on a 1% gel made up of low melting temperature agarose using *Hind*III digested λ DNA as markers, and the DNA extracted from the 4 appropriate gel slices, and each fraction dissolved in 20 µl of 0.1 mM EDTA. 1 µl of each DNA fraction was used for dot blot analysis (numbered 1-4), and hybridised using [<sup>35</sup>S]-phosphorylated 14-mer 1 as probe. The details of all procedures are given in Methods, Chapter 2.





However, this size selection provided sufficient DNA enriched for the *Hind*III fragment of interest for construction of a new library. By carrying out this size selection prior to cloning an estimated 80% of the DNA is eliminated.

#### 5.6 TRIAL SCREENING USING THE FOURTEEN-MERS DIRECTLY AS HYBRIDISATION PROBES

As outlined in the introduction to this chapter, the synthetic oligonucleotides can be used either directly as hybridisation probes or as primers for synthesis of cDNA using messenger RNA as template. Being the simplest, the first of these approaches was tried initially in screening a library (constructed as detailed later in this chapter using the vector pBR322 in *E. coli*) of the enriched *B. amyloliquefaciens* DNA isolated as described in Section 5.5.

Several attempts were made using various hybridisation and washing conditions to screen a colony library using the kinased 14-mers as direct hybridisation probes. However, in all these trials, background hybridisation to all colonies was very high and difficult to remove using high stringency washing conditions. Control experiments also showed strong hybridisation of the probes to the DNA of *E. coli* (the cloning host) and also the DNA of plasmid pBR322 (the vector used in construction of the library).

This result was somewhat disappointing and indicated that the second approach considered, that of primer extension using the fourteen-mers to prime cDNA synthesis from the messenger RNA of *B. amyloliquefaciens* would be required.

### 5.7 PRIMER EXTENSION TRIALS

In preparing an extended cDNA probe using the fourteen-mers as primers it would be advantageous to use as template, an RNA preparation in which the level of mRNA specific for the alkaline protease is high.

As has been discussed previously (see Chapter 1) earlier work in this laboratory has demonstrated that by use of appropriate incubation conditions, the capacity of *B. amyloliquefaciens* cells for rifampicin insensitive protease production can be modulated. Although the proposed mRNA pool for alkaline protease was not demonstrable in the translation work presented in Chapter 3, differences in mRNA levels may be demonstrated using the synthetic fourteen-mers in primer extension reactions using as template, RNA isolated under various conditions. To test this, two sets of conditions seemed convenient for RNA isolation (i.e. those used in Chapter 3) for use in primer extension trials. The two samples of RNA used were:

- (a) from washed cells incubated for seventy-five minutes in high amino acids medium, after which time the messenger RNA level for alkaline protease is expected to be minimal - designated 75 Haa RNA.
- (b) from washed cells incubated as for (a) in high amino acids medium, then for a further sixty minutes in low amino acids medium, after which time the messenger RNA level for alkaline protease is expected to be high - designated 60 Laa RNA.

It can reasonably be predicted that the major specific

primer extension products resulting when 60 Laa RNA is used as template will be more abundant than those resulting from the 75 Haa RNA sample.

The details of the washed cell experiments are given in Methods, Chapter 2. The RNA was isolated from whole cells using the extraction procedure discussed in Chapter 3 (Section 3.3) and partially purified on formamide-sucrose gradients as detailed in Section 3.10.

Primer extension reactions were carried out with each of the two fourteen-mers, using as template partially purified messenger RNA (in Fraction 2 RNA, see Section 3.10) isolated from *B. amyloliquefaciens* cells incubated under the two sets of conditions detailed above.

Preliminary reactions were carried out using various levels of RNA and fourteen-mers in various ratios. The set of conditions giving the most consistent results are detailed in Figure 5.3 and in Methods, Chapter 2.

Several points can be noted from the autoradiogram of the primer extension products shown in Figure 5.3. Firstly, the largest major extension products in Tracks 2, 3, 5 & 6 (arrowed) are more abundant in the 60 Laa RNA reactions when compared to the 75 Haa RNA reactions. The two major products synthesised when 14-mer 2 is used as primer are larger than those resulting from priming with 14-mer 1. This is consistent with specific priming of both oligomers on the messenger RNA, 14-mer 2 binding further towards the 3' end of the template. These major products are in excess of 400 bases in length as estimated from *Hinf* digested pBR322 used as markers in other trials. In the control samples, in which

### FIGURE 5.3

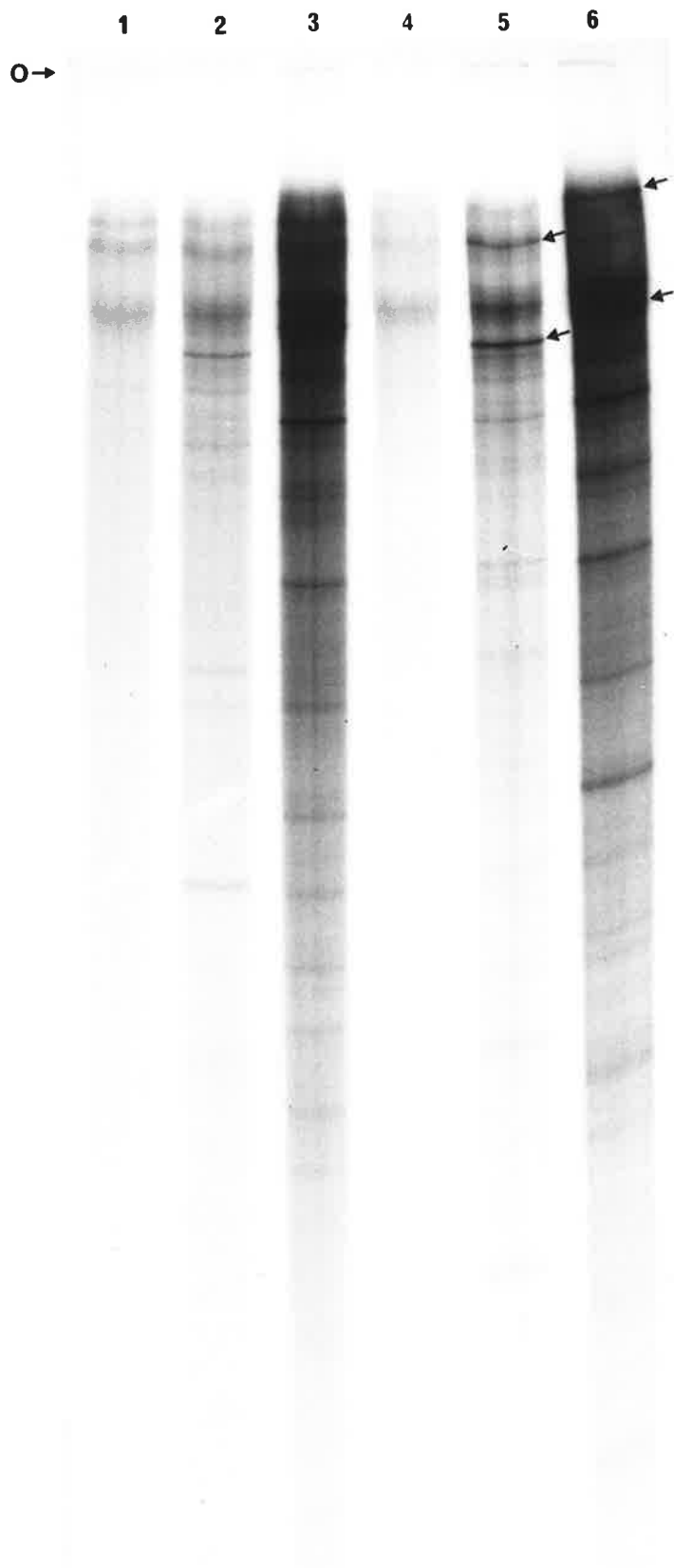
#### EXTENDED cDNA PRODUCTS USING 14-MERS 1 & 2 AND

#### 75 HAA AND 60 LAA RNA OF *B. AMYLOLIQUEFACIENS*

Extended cDNA products were synthesised using 0.5 µg of the appropriate oligonucleotide primer in reactions composed and carried out as described in Methods, Chapter 2, with 5 µg of each mRNA sample of *B. amyloliquefaciens* partially purified on formamide-sucrose gradients as detailed in Chapter 3, used as indicated below. 2 µl samples of the reactions were electrophoresed on a 6% polyacrylamide sequencing gel containing 7 M urea and autoradiographed for several hours at 4°C. The details of all procedure are given in Methods, Chapter 2.

#### TRACKS

1. 75 Haa RNA no added primer (control)
2. 75 Haa RNA, 14-mer 1
3. 75 Haa RNA, 14-mer 2
4. 60 Laa RNA, no added primer
5. 60 Laa RNA, 14-mer 1
6. 60 Laa RNA, 14-mer 2





no 14-mer primer was added there is a background range of cDNA products resulting from self priming. The major products in the other tracks are distinct from those in the controls, indicating that in the presence of the 14-mers, specific priming occurs.

This information is consistent with the major products being specific cDNA extension products from the alkaline protease messenger RNA. They are large enough to enable high stringency screening of a colony library which will alleviate problems of background hybridisation. The presence of two major products suggests premature termination of extension at a specific point, perhaps due to secondary structure formation during the reaction. It may also be due to priming on two related messenger RNA's. What is important however, is to test the specificity of hybridisation of these products prior to screening of a colony library.

#### 5.8 SOUTHERN ANALYSIS OF GENOMAL DNA USING PRIMER-EXTENDED cDNA

If the cDNA extension products discussed in Section 5.7 are specific for the alkaline protease gene, then in southern analysis of *B. amyloliquefaciens* DNA, they should hybridise with the same restriction fragments as were detected in the earlier work using the fourteen-mers directly as hybridisation probes. To test this the experiments described in Section 5.4 were repeated, using this time the primer extension products from each fourteen-mer when 60 Laa RNA was used as template, as hybridisation probes. These are designated

cDNA 1 and cDNA 2.

The results are given in Figure 5.4 and showed that the same fragments are hybridised to the extended probes as were detected when the fourteen-mers were used directly. The specificity of these probes implies that the major products are related to each other. That is, the mixture of cDNA products was used as a probe, and resulted in hybridisation largely to single fragments in each digest. Assuming under the washing conditions used that only the major products contribute significantly to the result then it may reasonably be suggested that they are related, as only a single restriction fragment is detected.

The result with cDNA probe 1 is very clear, with single fragments from each digest being detected. The cDNA probe 2 hybridised to other material but also showed strong hybridisation to fragments of the same size in each digest as cDNA probe 1. This suggests that this hybridisation is specific for the alkaline protease gene.

This result was very encouraging and permitted the immediate screening of a new colony library. As the extended material resulting when either primer was used hybridised to common restriction fragments, only one needed to be used for the initial screening and the material resulting from extension using 14-mer 1 as primer was used (i.e. cDNA probe 1) as the result was somewhat clearer.

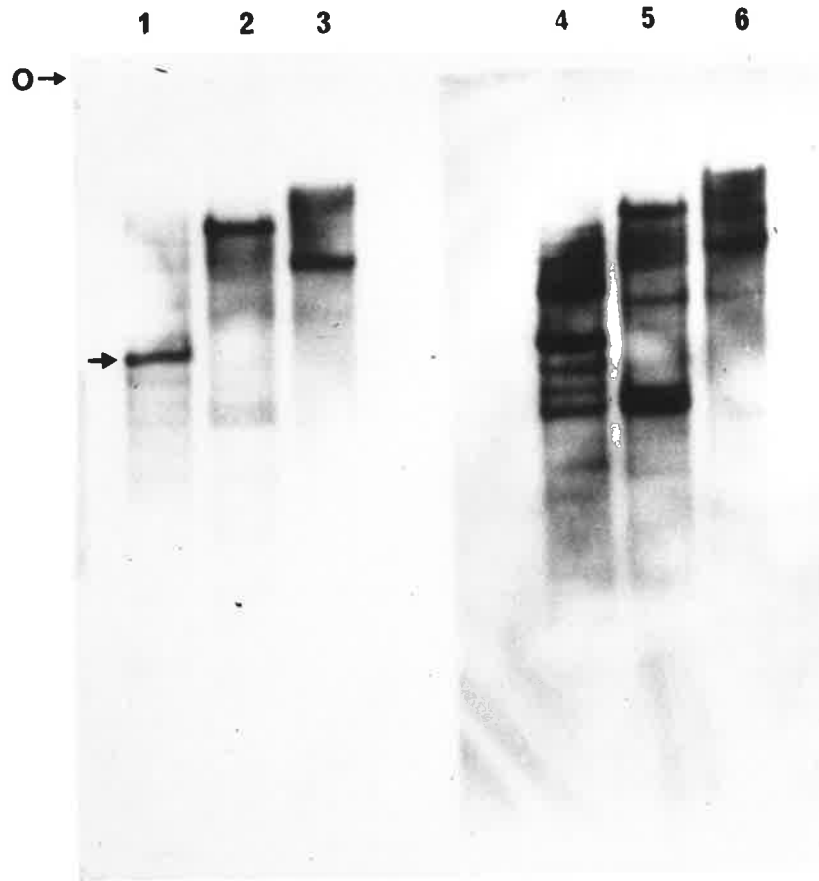
FIGURE 5.4

SOUTHERN ANALYSIS OF GENOMAL DNA OF *B. AMYLOLI-*  
*QUEFACIENS* USING PRIMER EXTENDED cDNA's 1 & 2  
PROBES

DNA of *B. amyloliquefaciens* (10 µg) was digested with *Hind*III, *Eco*RI or *Bgl*II. The reactions were split into two and samples loaded on separate Tracks of a 1% agarose gel and electrophoresed. After transfer to nitrocellulose the filter was bisected and probed with either cDNA 1 or cDNA 2 (synthesised using 14-mer 1 or 2 and 60 Laa RNA as detailed in 5.7) and autoradiographed -80°C 1 h. Details of all reactions and procedures are given in Methods, Chapter 2.

TRACKS

- |                                 |   |              |
|---------------------------------|---|--------------|
| 1. <i>Hind</i> III digested DNA | } | cDNA probe 1 |
| 2. <i>Eco</i> RI digested DNA   |   |              |
| 3. <i>Bgl</i> II digested DNA   |   |              |
| 4. <i>Hind</i> III digested DNA | } | cDNA probe 2 |
| 5. <i>Eco</i> RI digested DNA   |   |              |
| 6. <i>Bgl</i> II digested DNA   |   |              |



### 5.9 CONSTRUCTION AND SCREENING OF A COLONY LIBRARY USING A PRIMER EXTENDED PROBE (cDNA PROBE 1)

The enriched *B. amyloliquefaciens* DNA prepared as described in Section 5.5 was used to construct a new colony library by ligation into *Hind*III digested pBR322 vector and transformation into *E. coli*. Four filters, each with several thousand colonies were prepared for high-density screening essentially as described by Hanahan & Meselson (1980) and probed with primer extended cDNA synthesised using the 60 Laa RNA preparation and 14-mer 1 as outlined in Section 5.7 (i.e. cDNA probe 1).

The results of the screening are shown in Figure 5.5. Many strong positive signals were obtained which theoretically, should represent clones of the same *Hind*III fragment of *B. amyloliquefaciens* DNA, of about 2.6 Kb in size. The use of high stringency washing conditions has eliminated the problem of background hybridisation to the colonies on the nitro-cellulose filters, seen in earlier attempts to screen a library using the 14-mers as direct hybridisation probes.

### 5.10 REPLATING OF POSITIVE RECOMBINANTS AND RESCREENING

Because of the high density of colonies on each filter, it was not possible in some cases to be sure of picking a single colony. Therefore, for eight of the strong positive signals selected at random, ten "pickings" were carried out in the region of each hybridisation signal, onto fresh plates in an ordered array. These colonies were then replica plated and rescreened using both sets of primer extended cDNA products as shown in Figure 5.6.

FIGURE 5.5

HIGH DENSITY SCREENING OF COLONY LIBRARY USING

CDNA PROBE 1

A colony library was prepared in *E. coli* MC 1061 using the plasmid vector pBR322 and the size fractionated *Hind*III digested DNA of *B. amyloliquefaciens* prepared in Section 5.5. Colony filters were prepared and screened by hybridisation with cDNA probe 1 and autoradiographed at 4°C overnight. Details of the preparation of the colony library and screening and washing conditions are given in Methods, Chapter 2. Shown is an autoradiograph of one of the four colony filters screened in this manner.

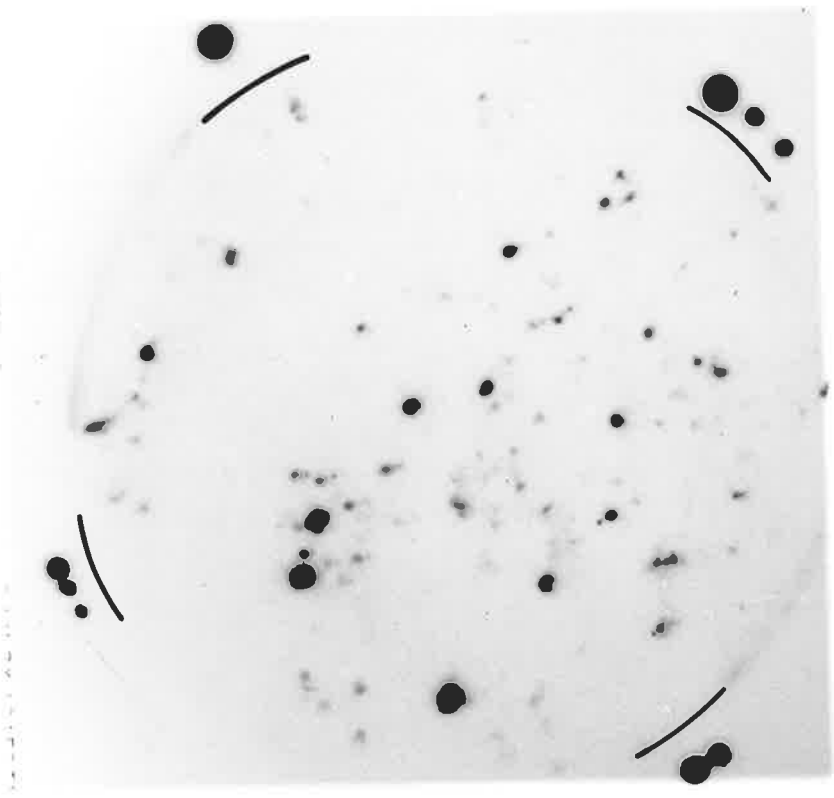


FIGURE 5.6

RESCREENING OF POSITIVE COLONIES WITH cDNA

PROBES 1 AND 2

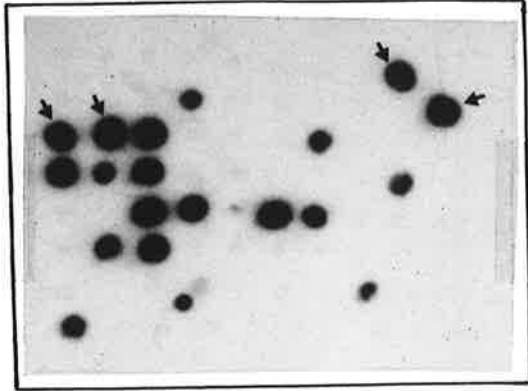
Duplicate filters from 80 colonies picked in an ordered array onto fresh plates were prepared and hybridised with cDNA probes 1 or 2 (made as detailed in 5.7) as described in Methods, Chapter 2. The filters were autoradiographed at  $-80^{\circ}\text{C}$  for 4 h.

1. Filter probed with cDNA 1
2. Filter probed with cDNA 2

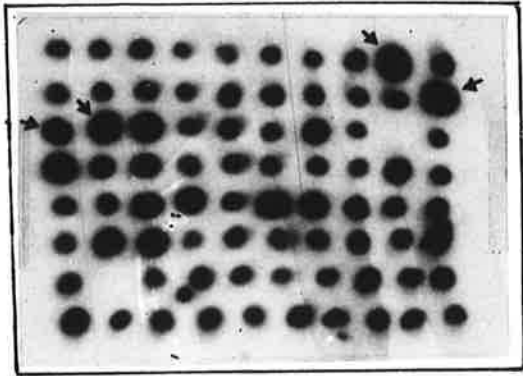
The four colonies chosen for further analysis are indicated.



1



2



Four of these clones as indicated showing strong hybridisation to both probes were further streaked out on fresh plates and single colonies used to prepare plasmid DNA using the miniprep procedure given in Methods, Chapter 2.

Restriction analysis of these four plasmid DNA preparations confirmed that they all represented pure clones of a single *Hind*III fragment of the correct size. Glycerol stocks of these clones were prepared as described in Methods, Chapter 2 and stored at  $-80^{\circ}\text{C}$ . Plasmid DNA of each clone was finally screened using the DNA dot-blot procedure, using [ $^{32}\text{P}$ ]-phosphorylated 14-mer 1 as a direct hybridisation probe as shown in Figure 5.7. All clones clearly hybridise very strongly to 14-mer 1 probe and the pBR322 controls are negative.

#### 5.11 PARTIAL RESTRICTION MAPPING OF PLASMID CLONE pBAP 1 AND SOUTHERN ANALYSIS

It is clear from the previous results that the four clones selected for dot-blot analysis are in fact the same. Therefore, a single clone designated pBAP 1 was selected for further characterisation. Pure plasmid DNA of this clone was prepared using the large scale procedure described in Methods, Chapter 2. Prior to DNA sequence analysis to confirm the identity of this clone, some limited restriction mapping and Southern analysis was carried out to locate within the insert, a suitable restriction fragment for immediate sequencing.

Samples of plasmid DNA of the clone were digested with

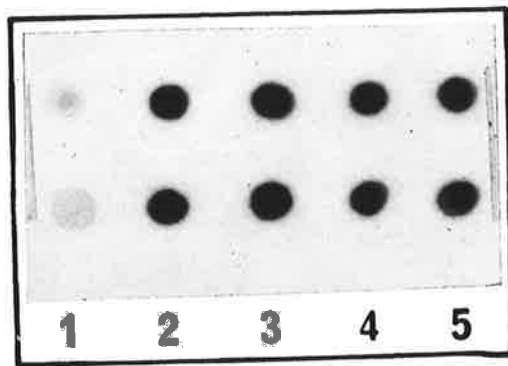
## FIGURE 5.7

### DNA DOT BLOT ANALYSIS OF PLASMID DNA'S OF CLONES 1-4

A dot blot filter was prepared with duplicate 1  $\mu$ g samples of each plasmid DNA and including vector plasmid pBR322 as control. The filter was hybridised with [ $^{32}$ P]-phosphorylated 14-mer 1 and autoradiographed 1 h at 4°C. The details of the procedures and probe preparation are given in Methods, Chapter 2.

#### TRACKS

1. pBR322 DNA
2. Clone 1 DNA
3. Clone 2 DNA
4. Clone 3 DNA
5. Clone 4 DNA



a range of restriction enzymes as described in Figure 5.8 and after Southern transfer, probed with the kinased 14-mer 1 probe.

From the gel of the digests it can be seen that these enzymes cleave the insert DNA of the clone infrequently.

A range of different enzymes were tried as shown in Figure 5.9 (a) & (b). In this case the digests were probed by Southern analysis with each of the 14-mers as direct hybridisation probes. This information, along with other combinations of digests (data not shown) allowed the construction of a partial restriction map of the clone as shown in Figure 5.10.

The Southern blot of Figure 5.9 shows that both 14-mers hybridise in all cases to the same restriction fragments. The smallest of these, a *Hind*III/*Pvu*II fragment of approximately 0.8 Kb in size is the fragment of choice for immediate sequence characterisation.

## 5.12 SEQUENCE CONFIRMATION OF THE IDENTITY OF PLASMID CLONE

### pBAP 1

Final confirmation that this plasmid clone (pBAP 1) is that for the alkaline protease gene requires direct demonstration by sequence analysis that the DNA in this clone codes for the protein sequence of the enzyme. Sequencing of the small *Hind*III/*Pvu*II fragment was carried out using the dideoxy method of sequencing single stranded M 13 clones as described in Methods, Chapter 2. In this case, the restriction fragment was purified, redigested with either *Sau*3A or *Hpa* II and these smaller fragments

## FIGURE 5.8

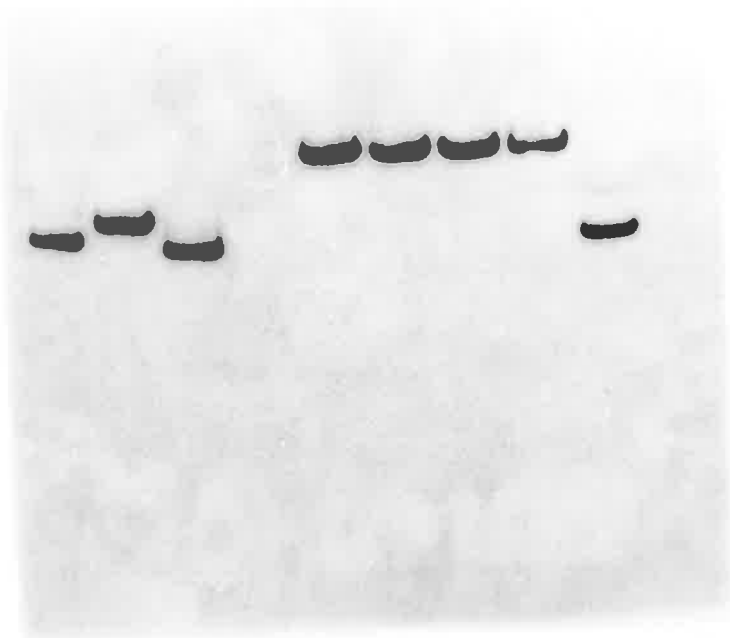
### SOUTHERN BLOT ANALYSIS OF pBAP1 USING [<sup>32</sup>P]- PHOSPHORYLATED 14-MER 1 AS PROBE

Plasmid DNA (1 µg) of pBAP1 was digested with the range of restriction enzymes indicated below, electrophoresed on a 1% agarose gel, transferred to nitrocellulose and hybridised with [<sup>32</sup>P]-phosphorylated 14-mer 1. *Hind*III digested λ DNA was included to provide standard markers. Details of all procedures are provided in Methods, Chapter 2.

#### TRACKS

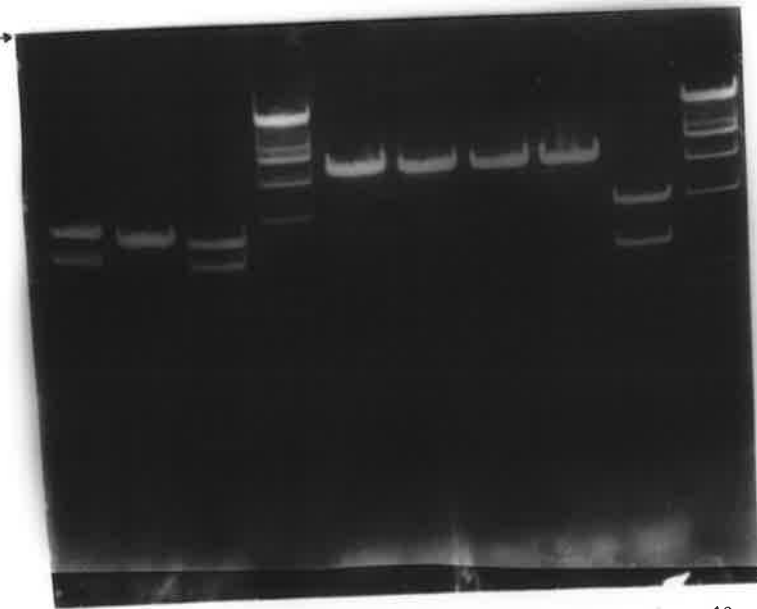
1. *Hind*III/*Sal*I digested pBAP1
2. *Eco*RI/*Sal*I digested pBAP1
3. *Pst*I digested pBAP1
4. *Hind*III digested λ DNA markers
5. *Bgl*III digested pBAP1
6. *Sal*I digested pBAP1
7. *Hind*III/*Pst*I digested pBAP1
8. *Eco*RI/*Sal*I digested pBAP1
9. *Hind*III/*Sal*I digested pBAP1
10. *Hind*III digested λ DNA markers

0→



1 2 3 4 5 6 7 8 9 10

0→



1 2 3 4 5 6 7 8 9 10

FIGURE 5.9

SOUTHERN BLOT ANALYSIS OF pBAP1 USING [<sup>32</sup>P]-  
PHOSPHORYLATED 14-MERS 1 AND 2 AS PROBES

DNA (1 µg) of pBAP1 or the purified 2.6 Kb insert of pBAP1 was digested with the range of restriction enzymes indicated below, electrophoresed on a 1% agarose minigel and transferred to nitrocellulose. The filter was hybridised separately with [<sup>32</sup>P]-phosphorylated 14-mer 1 or 2, boiling the filter for 5 min between experiments (autoradiography of the boiled filter showed that all previously hybridised probe is removed). Autoradiography was for 2 h, at 4°C. All details of procedures are provided in Methods, Chapter 2.

TRACKS

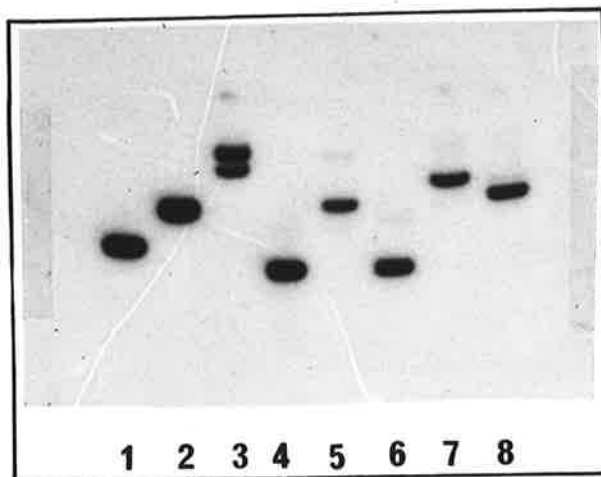
1. *AccI* digested insert
2. *SmaI* digested insert
3. *SmaI* digested pBAP1
4. *PvuII* digested insert
5. *PvuII* digested pBAP1
6. *PvuI* digested insert
7. *PvuI* digested pBAP1
8. *HindIII* digested pBAP1

(a) = 14-mer 1 probe

(b) = 14-mer 2 probe



(a)



(b)

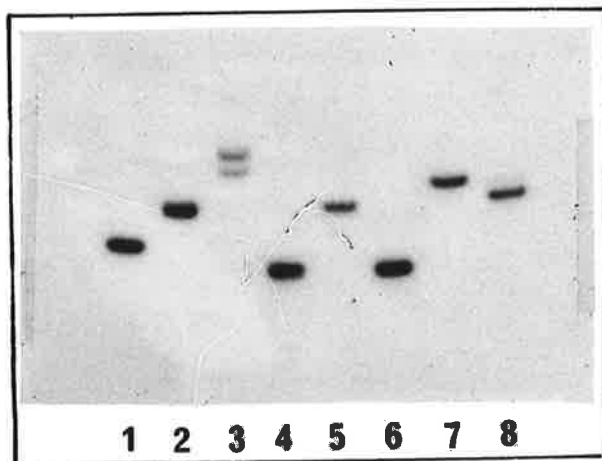
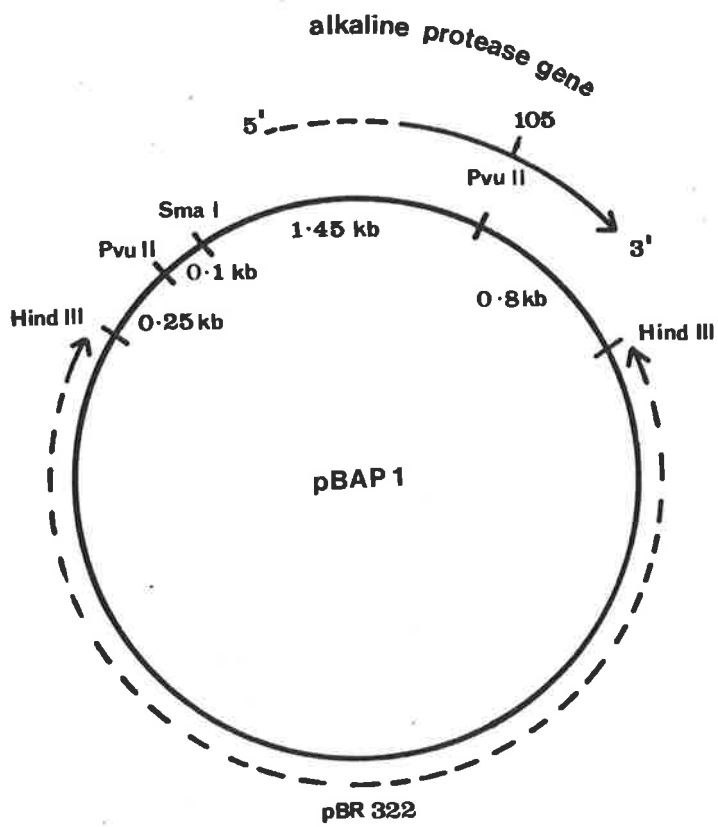


FIGURE 5.10

PARTIAL RESTRICTION MAP OF pBAP1

The restriction map was derived from the appropriate restriction enzyme digests of plasmid pBAP1. Fragment sizes were estimated by electrophoresis of the digests on 1% agarose gels, using *Hind*III digested  $\lambda$  DNA as molecular weight markers. Only restriction sites within the 2.6 Kb *Hind*III insert are indicated, and fragment sizes given. The orientation of the alkaline protease gene is indicated. The *Pvu*II site within the gene corresponds to amino acid 105 of the alkaline protease.



cloned into M13. All the details for these procedures are provided in Methods, Chapter 2. Sequencing directly from the *PvuII* site was also carried out after cloning into the appropriate M13 vector.

Part of the DNA sequence of the *HindIII/PvuII* site is shown in the sequencing gel of Figure 5.11. This stretch of DNA contains sequence corresponding to the synthetic oligonucleotide 14-mer 1 (as indicated) and confirms the identity of the pBAP 1 plasmid clone, as indicated below.

The DNA sequence and corresponding amino acid sequence from the *PvuII* site to a *HpaII* site is presented in Figure 5.12. This amino acid sequence (predicted by computer analysis of the DNA sequence) corresponds exactly to amino acids 105 to 145 of the published sequence of the alkaline protease (Markland & Smith, 1967). The result establishes that plasmid clone pBAP 1 contains DNA specific for the alkaline protease. From the position of the *PvuII* site within the 2.6 Kb insert (Figure 5.10) it can be deduced that the clone contains the entire gene sequence of alkaline protease.

### 5.13 FURTHER SEQUENCING OF THE ALKALINE PROTEASE GENE OF

#### *B. AMYLOLIQUEFACIENS*

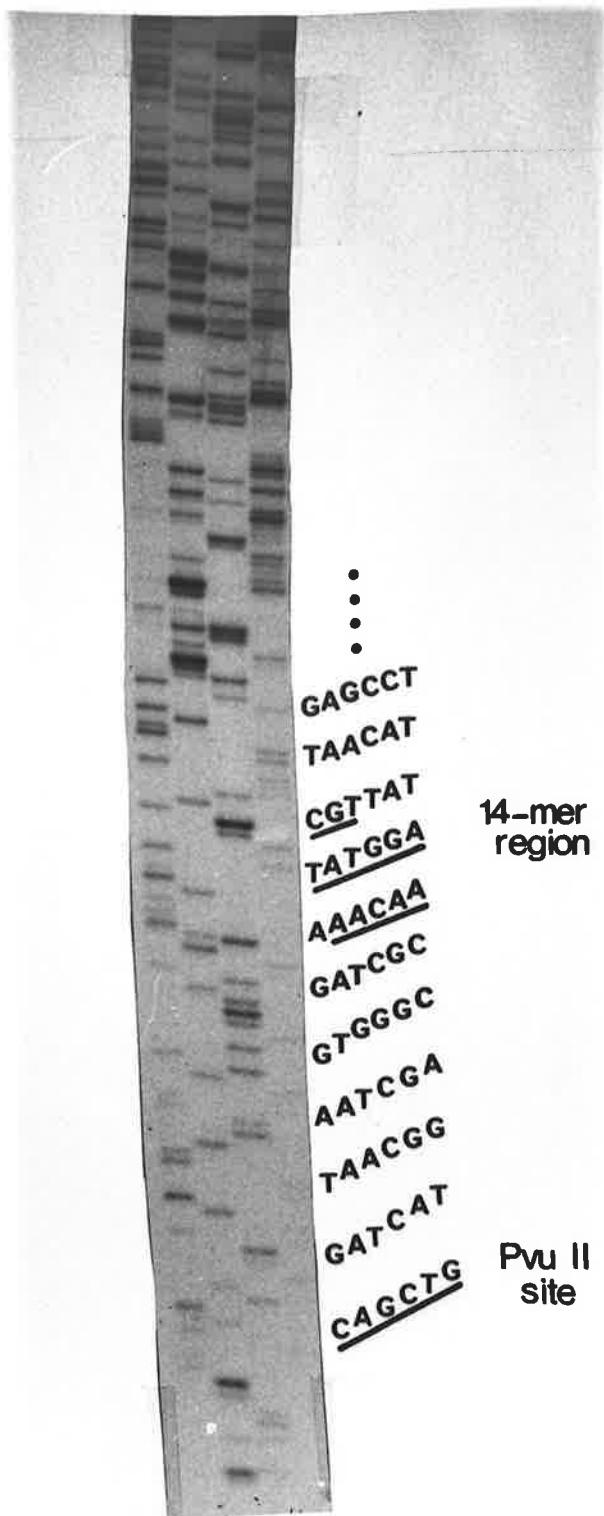
The complete DNA sequence of the alkaline protease gene is desirable to enable further studies of its structure and function as will be discussed in more detail later.

While further sequencing of the 2.6 Kb insert of pBAP 1 was in progress, and the writing of this thesis commenced, Wells *et al.* (1983) reported the cloning and complete sequence analysis of the alkaline protease gene of

FIGURE 5.11

PARTIAL DNA SEQUENCE ANALYSIS OF THE ALKALINE  
PROTEASE GENE OF *B. AMYLOLIQUEFACIENS*.

A fragment of DNA (a *Pvu*II/*Hpa*II fragment) from plasmid clone pBAP1 was cloned into the appropriate M13 vector and sequenced using the chain-termination method of Sanger *et al.* (1977). Samples were electrophoresed on a 6% acrylamide-urea sequencing gel until the bromophenol blue tracker dye had migrated to the bottom of the gel. Details of all preparations and procedures are given in Methods, Chapter 2.



⋮

GAGCCT  
TAACAT  
CGTTAT  
TATGGA  
AAACAA  
GATCGC  
GTGGGC  
AATCGA  
TAACGG  
GATCAT  
CAGCTG

14-mer  
region

Pvu II  
site

A C G T

FIGURE 5.12

DNA SEQUENCE AND CORRESPONDING AMINO ACID

SEQUENCE OF A PVUII/HPAII SEGMENT OF pBAP1

DNA sequence analysis was carried out as described in Figure 5.11 and the amino acid sequence derived by computer analysis. The numbers refer to the corresponding amino acids in the published sequence of the alkaline protease of *B. amyloliquefaciens* (Markland & Smith, 1967).

	105					110			
	Ser	Trp	Ile	Ile	Asn	Gly	Ile	Glu	Trp
C	<u>AGC</u>	<u>TGG</u>	ATC	ATT	AAC	GGA	ATC	GAG	TGG
	PvuII								
		115					120		
	Ala	Ile	Ala	Asn	Asn	Met	Asp	Val	Ile
	GCG	ATC	GCA	AAC	AAT	ATG	GAC	GTT	ATT
			125					130	
	Asn	Met	Ser	Leu	Gly	Gly	Pro	Ser	Gly
	AAC	ATG	AGC	CTC	GGC	GGA	CCT	TCT	GGT
				135					140
	Ser	Ala	Ala	Leu	Lys	Ala	Ala	Val	Asp
	TCT	GCT	GCT	TTA	AAA	GCG	GCA	GTT	GAT
					145				
	Lys	Ala	Val	Ala	Ser				
	AAA	GCC	GTT	GCA	<u>TCC</u>	<u>GG</u>			
					HpaII				



*B. amyloliquefaciens*. The sequence data obtained thus far for pBAP 1 (which includes the entire sequence from the 3' *Hind*III site encompassing the putative terminator structure, to amino acid -7 (see Wells *et al.*, 1983) agrees completely with that of the above authors, and is therefore not reiterated here.

The implications of this for our future work are discussed in the concluding chapter of this thesis.

#### 5.14 DISCUSSION

The work presented in this chapter resulted in the successful isolation and partial characterisation of the gene for the alkaline extracellular protease of *B. amyloliquefaciens*.

Detailed study of the control of this gene (as will be discussed in more detail in the concluding chapter of this thesis) is currently in progress in this laboratory.

This gene was isolated using as a hybridisation probe, cDNA made using a 14-mer primer on a template of RNA isolated from *B. amyloliquefaciens*. The successful use of this probe confirms the presence of mRNA for the alkaline protease in RNA preparations from *B. amyloliquefaciens*, independently of the *in vitro* translation work presented in Chapter 3. This primer extension work (see Section 5.7) also strongly suggests that this mRNA is more abundant in RNA isolated from cells optimised for the apparent protease mRNA pool, than from cells in which this pool has been depleted (i.e. 60 Laa RNA v's 75 Haa RNA). The possible

implications of this for the work presented in Chapter 3 and for future work are discussed further in the following chapters.

CHAPTER SIX

PRELIMINARY HYBRIDISATION STUDIES  
OF ALKALINE PROTEASE mRNA PRODUCTION  
BY B. AMYLOLIQUEFACIENS

## 6.1 INTRODUCTION

Earlier work in this laboratory, as discussed in Chapter 1, had demonstrated the unusual response of protease production by *B. amyloliquefaciens* to inhibitors of transcription. An hypothesis was put forward (O'Connor *et al.*, 1978) to account for these findings, based on the concept of an apparent mRNA pool for protease. Of central interest in the work presented in this thesis was to determine whether the apparent protease mRNA pool exists, by examining protease mRNA production under various culture conditions. The successful isolation of the gene for the alkaline protease of *B. amyloliquefaciens*, as described in the previous chapter, placed these studies on a molecular basis. Changes in mRNA levels for this enzyme can now be examined directly by hybridisation analysis. Presented here are two preliminary experiments along this line of investigation, using the alkaline protease gene probe.

## 6.2 DOT BLOT ANALYSIS OF 75 HAA RNA AND 60 LAA RNA OF

### *B. AMYLOLIQUEFACIENS*

As was discussed in Chapter 3, the *B. amyloliquefaciens* RNA preparations designated 75 Haa RNA and 60 Laa RNA represent time points at which the apparent protease mRNA pool is minimal and approaching maximal respectively. The primer extension studies described in Chapter 5 using these RNA samples and the synthetic DNA oligonucleotides specific for the alkaline protease gene, suggested that, as expected, the mRNA for alkaline protease was more abundant in the 60 Laa RNA preparation.

To look directly at protease mRNA levels, the 75 Haa RNA and 60 Laa RNA samples were examined by dot blot analysis using part of the cloned alkaline protease gene as a hybridisation probe, as described in Figure 6.1. The probe used was a ss DNA M 13 clone of a *Sau3A* fragment of the alkaline protease gene contained in plasmid pBAP1 (refer to Chapter 5). This DNA probe was complementary to mRNA corresponding to amino acids 115 - 268 of the published sequence of alkaline protease (Markland & Smith, 1967).

Three levels of each RNA sample were used in this initial trial as shown in Figure 6.1. A progressive increase in hybridisation with the DNA probe is evident in the Laa RNA samples (Dots 4 - 6) whereas hybridisation to the 75 Haa RNA samples (Dots 1 - 3) is faint. The relative amounts of probe hybridised to each sample was estimated by cutting out the appropriate areas of the nitrocellulose filter and counting using Cherenkov radiation. At the largest amount of RNA used (20  $\mu$ g, Samples 3 and 6) an eight fold increase in the counts hybridised to 60 Laa RNA compared to counts hybridised to 75 Haa RNA was observed. Clearly, dot blot analysis must be carried out using a broader range of RNA levels. However, this early result establishes that the mRNA for alkaline protease is present in significantly larger amounts in 60 Laa RNA than in 75 Haa RNA; a finding consistent with our earlier work.

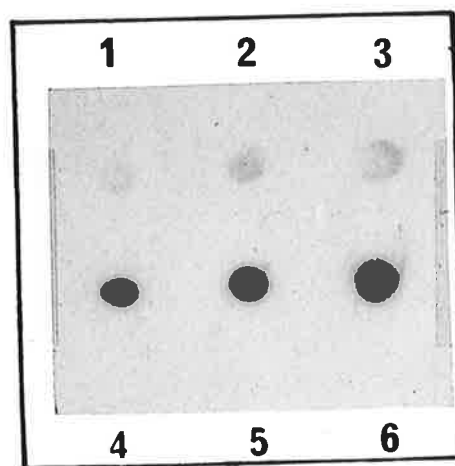
## FIGURE 6.1

### DOT BLOT ANALYSIS OF 75 HAA RNA AND 60 LAA RNA OF *B. AMYLOLIQUEFACIENS*

75 Haa RNA and 60 Laa RNA samples of *B. amyloliquefaciens* were isolated as described in Chapter 3 and Methods, Chapter 2. Various amounts of these RNA samples as indicated below, were transferred to nitrocellulose using the dot blot procedure. The filter was probed with a [<sup>32</sup>P]-labelled single stranded M13 probe complementary to mRNA corresponding to amino acids 115 to 268 of the alkaline protease, prepared using an M13 clone containing a *Sau*3A restriction fragment of the corresponding DNA. Details of all preparations and procedures are given in Methods, Chapter 2.

#### SAMPLES

1. 5 µg 75 Haa RNA
2. 10 µg 75 Haa RNA
3. 20 µg 75 Haa RNA
4. 5 µg 60 Laa RNA
5. 10 µg 60 Laa RNA
6. 20 µg 60 Laa RNA



### 6.3 NORTHERN BLOT ANALYSIS OF *B. AMYLOLIQUEFACIENS* RNA

#### TIME COURSE SAMPLES.

It was of interest to look at the size of the alkaline protease mRNA and to examine the changes in protease mRNA levels which might occur over a time course of incubation of *B. amyloliquefaciens* cells in low amino acids suspending medium. Accordingly, RNA was isolated as described in Figure 6.2, from cells of *B. amyloliquefaciens* which had been incubated for 75 min in high amino acids medium, and also following a further 20, 40, 60, 75 and 90 min incubation in low amino acids medium.

Assays of total protease and alkaline protease from cells at each time point were carried out as described by Love (1981), and showed protease production to be linear in both instances.

Samples of RNA (20 µg) from each time point were electrophoresed on a 1%-agarose-methylmercury gel, transferred to nitrocellulose and hybridised with the same mRNA-specific probe used in the previous section. The details and result of this experiment are shown in Figure 6.2.

The autoradiogram shows a marked increase in hybridisation to the 20 Laa RNA sample (Track 2) when compared to the 75 Haa RNA sample (Track 1), consistent with the increase in alkaline protease mRNA seen in Section 6.2 upon transfer of cells from high amino acids medium to incubation in low amino acids medium.

Particularly interesting (and potentially exciting) was the observation of two specific RNA bands hybridising with the alkaline protease gene probe. These two bands are positioned just below the 16S rRNA species (as measured using a UV photograph of the stained gel prior to transfer). These bands vary



## FIGURE 6.2

### NORTHERN BLOT ANALYSIS OF *B. AMYLOLIQUEFACIENS*

#### RNA TIME COURSE SAMPLES

6 x 40 ml lots of *B. amyloliquefaciens* cells were incubated for 75 min in high amino acids medium and further incubated in low amino acids medium as described in Methods, Chapter 2. RNA was prepared from 40 mls of cells for each time point (i.e., after 75 min in high amino acids medium, [75 Haa RNA], and after a further 20, 40, 60, 75, or 90 min in low amino acids medium [20-90 Laa RNA samples respectively]). 20 µg of each RNA sample was electrophoresed on a 1% agarose-methylmercury gel, visualised by ethidium staining, transferred to nitrocellulose and hybridised with the [<sup>32</sup>P]-labelled M13 probe used in Figure 6.1. The final washing of the nitrocellulose filter was for 30 min at 60°C in 0.2 X SSC. Details of all preparations and procedures are provided in Methods, Chapter 2.

#### SAMPLES

1. 75 Haa RNA
2. 20 Laa RNA
3. 40 Laa RNA
4. 60 Laa RNA
5. 75 Laa RNA
6. 90 Laa RNA



1 2 3 4 5 6

greatly in intensity, the upper one being only faintly observable. However, both appear to increase in hybridisation intensity up to the 60 Laa RNA sample (Track 4) and then to decrease. The difference in intensity is marked between the 75 Haa RNA and 20 Laa RNA samples (as mentioned previously) but in further samples the change in band intensities is only slight. The possible significance of this is discussed later in this chapter.

The presence of two species of RNA hybridising to the alkaline protease gene probe raises several interesting possibilities. *B. subtilis* produces both an extracellular and intracellular alkaline protease (subtilisin) which share approximately 50 percent homology at the amino acid level (Strongin *et al.*, 1978). An intracellular protease in *B. amyloliquefaciens* has been identified and partly characterised (Paton, 1979). The possibility arises that the gene probe used in the present work may hybridise to the mRNA's for two related proteases in *B. amyloliquefaciens*, giving rise to two bands on the Northern gel of Figure 6.2.

Another possibility is that two forms of mRNA for the extracellular alkaline protease are produced, perhaps by initiation of transcription at different start sites. This clearly needs to be investigated further by *in vitro* transcription analysis. However, on the basis of the relatively large amounts of extracellular alkaline protease produced by *B. amyloliquefaciens*, it can reasonably be suggested that at least the major hybridising band in this experiment is specific for the mRNA of the extracellular enzyme.

#### 6.4 DISCUSSION

The preliminary hybridisation studies of alkaline protease mRNA production by *B. amyloliquefaciens* presented here, show that the levels of this mRNA alter in response to various incubation conditions.

These results demonstrate that the level of alkaline protease mRNA in cells incubated for 75 min in high amino acids medium is very low, consistent with the repressive effect of amino acids discussed in Chapter 1. Upon transfer of these cells to low amino acids medium and further incubation, the level of alkaline protease mRNA was shown to increase dramatically.

During the first 20 min of incubation in low amino acids medium the elevation of alkaline protease mRNA levels is marked (compare Tracks 1 and 2 of Figure 6.2), with only a relatively small increase occurring during further incubation in low amino acids medium. It appears that transcription is very rapid during the initial period of incubation in low amino acids medium. This clearly needs to be examined in greater detail.

It was mentioned earlier that alkaline protease production was shown to be linear during the time course experiment described in Section 6.2. This information, coupled with the rapid build-up of mRNA seen during the first 20 min of incubation in low amino acids medium, suggests that all of the mRNA transcribed is not utilized immediately for translation.

These preliminary experiments, although indicative of changes in mRNA levels for alkaline protease say nothing directly about the stability of this mRNA encompassed in the

concept of a "reserve" pool of mRNA. Clearly, a more detailed hybridisation analysis of alkaline protease mRNA production must be carried out before conclusions can be drawn as to the possible nature of the apparent mRNA pool for protease discussed in Chapter 1. For example, time course experiments, in the presence and absence of rifampicin treatment of cells will enable putative stable mRNA's to be identified.

The possible significance of the two hybridising RNA's seen in Figure 6.2 awaits *in vitro* transcription studies and is discussed further in the following chapter.

The preliminary work presented here serves largely to point the way for a more detailed study of alkaline protease mRNA production by *B. amyloliquefaciens* at the molecular level. This future work, is discussed in more detail in the following chapter.

CHAPTER SEVEN

FINAL SUMMARY AND DISCUSSION

## FINAL SUMMARY AND DISCUSSION

The work presented in this thesis provides the basis for the further study of several aspects of the synthesis and secretion of the extracellular alkaline protease of *B. amyloliquefaciens*. Earlier work in this laboratory demonstrated the peculiar characteristics of protease production by this organism. The size of the apparent mRNA pool for protease disclosed in this work was only measurable in terms of the total protease (of either major species) produced by cells in the presence of an inhibitor of transcription (rifampicin or actinomycin D). The successful isolation of the gene for the alkline protease (described in Chapter 5) gives the means to place these studies, for this enzyme, on a molecular basis.

In Chapter 6 preliminary hybridisation studies of alkaline protease mRNA production were described. These experiments were supportive of the concept of an mRNA pool for this enzyme, and indicated the direction for a detailed examination of this complex phenomenon. We are now in a position to characterise the apparent alkaline protease mRNA pool.

The studies of Love (1981) on the production of alkaline and neutral proteases by *B. amyloliquefaciens* led to the implication that the genes for these two enzymes are separately controlled. A parallel study of neutral protease mRNA production by this organism is obviously desirable, as both enzymes contribute to the unusual phenomena described in our earlier work.

In respect of this, it was mentioned in the discussion of Chapter 4, that the clone isolated in the work described in that chapter was possibly related to the neutral protease.

We intend in this laboratory to purify the neutral protease from the culture supernatant of *B. amyloliquefaciens*. This will enable further examination of the above clone and if necessary the isolation of the gene for the neutral protease using an analogous approach to that described in Chapter 5.

The successful use of the primer extended probes in isolation of the alkaline protease gene (Chapter 5) and the preliminary studies of protease mRNA production carried out using the gene probe (Chapter 6) indicated that cells of *B. amyloliquefaciens* incubated in low amino acids medium contain elevated levels of alkaline protease mRNA; (ie., 60 Laa RNA). On a preparative scale, the cloned gene can be used in a hybrid selection technique similar to that described in Chapter 4, to isolate large amounts of pure alkaline protease mRNA. This will enable *in vitro* translation and transport studies to be undertaken using the *E. coli* cell-free system described in Chapter 3. Use of pure mRNA may clarify some of the work described in that chapter, and in this respect, the antiserum prepared to the alkaline protease may be useful. The translatability of the mRNA may be examined afresh, using the *E. coli* cell-free system or perhaps systems supplemented with cell-free extracts of *B. amyloliquefaciens*.

The DNA sequence of the alkaline protease gene (Wells *et al.*, 1983) predicts that the enzyme is synthesised as a preprotein with a signal peptide and a region of about 75 amino acids of unknown function between the signal peptide and the start of the mature protein. The proposed signal peptide appears typical of other *Bacillus* secretory proteins for which the signal peptides are known (Wells *et al.*, 1983). *In vitro*



translation and transport studies using the pure mRNA may reveal a translational precursor product, enabling a more detailed examination of its function.

The production of protease in the absence of transcription, and the changes in the apparent mRNA pool size demonstrated in earlier work (O'Connor *et al.*, 1978) were explained by proposing that two forms of mRNA were produced. It was envisaged that during transcription mRNA was supplied directly for translation and that at the same time a reserve of non-translatable mRNA was built up, the latter constituting the mRNA pool. In the absence of transcription the reserve was considered to be converted to translatable mRNA.

Of fundamental interest in all of this work is an understanding of the control of protease mRNA production, and of regulation of the translation of the apparent mRNA pool. Clearly two levels of control are implicated in this system if the concept of a reserve mRNA pool, not immediately translatable, is correct. The first level, that of transcriptional control of expression of the alkaline protease gene is self-evident, and will be discussed briefly. The second level of control, implicit in the concept of a reserve mRNA pool, and therefore directly pertinent to the work presented in this thesis is a translational control mechanism of some kind.

Consensus sequences that signal the initiation of transcription and translation in *B. subtilis* have been determined, (Moran *et al.*, 1982), which correspond closely to those attributed to *E. coli*. From the complete DNA sequence of the alkaline protease gene of *B. amyloliquefaciens* (Wells *et al.*, 1983), putative promoter and ribosome binding site regions

have been assigned. These workers also found that the cloned gene was not expressed in *E. coli*, but very efficiently expressed when cloned into *B. subtilis*. It was suggested that expression required the presence of specific sigma factors from *B. subtilis*. These factors, subunits of bacterial RNA polymerases, modulate the promoter recognition specificity of the core polymerase (Losick & Pero, 1981; Johnson *et al.*, 1983). *In vitro* transcription and S<sub>1</sub> nuclease mapping studies are required to confirm the putative promoter region and sigma factor specificity of the cloned alkaline protease gene. As was mentioned previously, we are in a position to purify the mRNA and in respect of the above work, direct RNA sequencing using specific synthetic primers will be useful in characterisation of transcripts.

The second level of control, while dependent on direct confirmation of the existence of a reserve mRNA pool, and therefore speculative at this stage, requires that translation of the mRNA is delayed by some mechanism, i.e., that translational control is operating. Questions that need to be answered include; does the apparent reserve mRNA pool exist as uniquely stable mRNA and if so, how does this situation arise?

It is interesting to speculate that translation of the mRNA is blocked through a system similar to the SRP complex and docking protein demonstrated in eukaryotes (Walter & Blobel, 1983) and that the mRNA is stabilised by such an interaction, or through the concomitant formation of a stable secondary structure, or a combination of both processes. A system similar to the SRP mechanism has been recently post-

ulated to exist in *E. coli*, (reviewed by Silhavy *et al.*, 1983). The qualification required for the reserve mRNA pool concept is that the translational block is not dependent on availability of docking sites for co-translational secretion, but is achieved by a separate control mechanism. It has not been established for protease production by *B. amyloliquefaciens*, whether these proteins are synthesised on membrane-associated ribosomes (as has been suggested for other secretory proteins in *B. subtilis* (Smith *et al.*, 1979, Mazars *et al.*, 1983), or via another mechanism. The site of exoenzyme synthesis in *B. amyloliquefaciens* has been the subject of a recent study in this laboratory (Love, 1981) and requires further examination.

This delayed translation proposal may also be likened to the translational attenuation models put forward by Hahn *et al.* (1982) to account for the regulation of synthesis of the gene product of *erm C*, a plasmid gene, which specifies resistance to macrolide-lincosamide-streptogramin B antibiotics. The product of the *erm C* gene is an inducible rRNA methylase. The model put forward by these workers postulated that alternative inactive and active conformational states of the *erm C* mRNA are modulated by erythramycin-induced ribosome-stalling during translation of a leader peptide. Rapid induction of methylase synthesis was shown to occur in the presence of antibiotic, and using rifampicin or streptolydigin in minicell studies, induction was shown to occur in the absence of transcription, and was accompanied by a dramatic and specific increase in mRNA functional stability.

This methylase synthesis in the absence of transcription

is similar to the rifampicin-actinomycin D-insensitive production of protease by *B. amyloliquefaciens* demonstrated in earlier work in this laboratory which led to the concept of an apparent mRNA pool for this enzyme (O'Connor *et al.*, 1978).

A similar model to that put forward for regulation of the *erm C* system (Hahn *et al.*, 1982) may be proposed to operate here. Translation of the protease mRNA may be regulated by ribosome-stalling during translation of a leader peptide, resulting in a pool of mRNA not immediately translatable. Indeed, the complete DNA sequence of the alkaline protease gene of *B. amyloliquefaciens* (Wells *et al.*, 1983) revealed that if the signal peptide is typical (as appears to be the case) then encoded in the sequence is a region of about 75 amino acids of unknown function between the end of the signal peptide and the mature protein. These workers determined that this region strongly resembles a propeptide structure, but it could conceivably also operate in a manner analogous to the leader peptide in the *erm C* system discussed above.

Several other instances of translation level control were cited by Hahn *et al.* (1982) and another very recent example is provided by MacDonald *et al.* (1984) in a study of the expression of the bacteriophage T4 *soc* gene. This gene is transcribed both early and late, but is translated only late. The inhibition of *soc* translation from the long early transcripts was explained by formation of a hairpin in the RNA that sequesters the *soc* ribosome-binding site. The transcript initiated at the late promoter cannot form this hairpin and is therefore translated.

Analysis of *B. amyloliquefaciens* RNA samples by Northern blot (Chapter 6, Figure 6.2) showed the presence of two RNA bands hybridising to gene probe. It was suggested that two forms of the RNA may be present, arising by transcription from different initiation sites. It is tempting to speculate that a translational control system similar to that described for the *soc* gene in bacteriophage T<sub>4</sub> operates for alkaline protease mRNA production, involving the synthesis of two transcripts, one able to form a hairpin structure which blocks translation initiation. Again, *in vitro* transcription studies are required to examine this possibility further.

In final summary, we are now in a position to examine in detail, at the molecular level, this most interesting control situation which is evident in the production of extracellular protease by *B. amyloliquefaciens*.

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