

**PURIFICATION AND MOLECULAR ANALYSIS
OF THE *KLUYVEROMYCES LACTIS*
LINEAR KILLER PLASMIDS**

**Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester**

by

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ABSTRACT

The killer phenotype of the lactose-utilising yeast *Kluyveromyces lactis* is dependent upon the presence of two linear, dsDNA plasmids termed pGKL1 (K1) and pGKL2 (K2). Both plasmids have terminal inverted repeat sequences (TIRs) and terminal proteins (TPs) linked to their 5' ends. Structurally similar genetic elements, termed invertrons, which include human adenovirus and *Bacillus subtilis* bacteriophage ϕ 29, have been shown to replicate their DNA by a protein-primed mechanism. The cytoplasmic location of K1 and K2, and the finding of putative plasmid-encoded family B DNA polymerases suggested that they encode their own protein-primed replication system.

Although many invertrons have been described in various organisms, with the exception of adenovirus and bacteriophage ϕ 29, little is known about their biology. Difficulties arise in studying killer plasmid gene function as their structure cannot be readily modified by *in vitro* manipulation due to the presence of protein-linked termini. Furthermore, the killer plasmids appear to encode their own transcription mechanism, and their genes are not correctly expressed by the host transcription system when cloned into yeast on conventional nuclear located circular vectors. Although *in vivo* homologous recombination studies have progressed our understanding of the killer plasmids, a method for their *in vitro* modification and transformation has not been developed, and biochemical and molecular analysis has progressed slowly.

To advance study in these areas, a large scale procedure for the purification of the *K. lactis* linear killer plasmids, with intact terminal proteins (TPs), was developed using gentle NP40-induced lysis of yeast spheroplasts, and standard chromatography methods. A 15 kDa protein, thought to be TRF1, co-purified with the killer plasmids. The TPs were shown to be intact by electrophoresis of the killer plasmids on agarose gels, analysis of terminal restriction fragments (TRFs) by PAGE, and direct visualisation of iodinated TPs by SDS-PAGE. New molecular weight estimates of 26.5 and 30.5 kDa were determined for the K1 and K2 TPs, respectively. Analysis of the K2 TP-DNA linkage suggested that the phosphodiester bond between the K2 TP and its DNA is likely to be via a tyrosine residue.

The new isolation procedure for K1 and K2 allowed further investigations to be performed which were not possible with previous purification methods. Thus, attempts were made to reintroduce the killer plasmids back into the host strain, and to determine the amino acid sequence of the TPs. The data obtained in these experiments have provided a platform from which further studies on killer plasmid biology can be made.

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ABBREVIATIONS

A	absorbance
A	adenine, or adenosine
Ad pol	adenovirus DNA polymerase
ARS	autonomously replicating sequence
bp	base pairs
C	cytosine, or cytidine
CBB	Coomassie Brilliant Blue stain
DEAE	diethylaminoethyl
DBP	DNA binding protein
DHBV	duck hepatitis B virus
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
dNTP	deoxyribonucleoside triphosphate of A, C, G, or T
EDTA	ethylenediamine tetraacetic acid
FPLC	fast protein liquid chromatography
g	gravitational force
dsDNA	double-stranded DNA
DTT	dithiothreitol
EtBr	ethidium bromide
G	guanine, or guanosine
GSHV	ground squirrel hepatitis virus
GST	glutathione S-transferase
HBV	hepatitis B virus
K	rotor speed ($\times 10^3$ r.p.m.)
kb	kilobases
kDa	kiloDalton
L	litre
M_r	molecular weight
mtDNA	mitochondrial DNA
mRNA	messenger RNA
MOPS	morpholinopropane sulphonic acid
NMP	nucleoside monophosphate of A, C, G, or T
NMWC	nominal molecular weight cut-off
NP40	Nonidet P40
NTP	nucleoside triphosphate of A, C, G, or T
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
PP	primer protein
pTP	precursor terminal protein
Q-water	Milli-Q reagent water system (Millipore) purified water
ρ	density
RNA	ribonucleic acid
RNase A	ribonuclease A
RNase H	ribonuclease H
sarkosyl	<i>N</i> -Dodecanoylsarcosinate
SDS	sodium dodecyl sulphate (sodium lauryl sulphate)
ssDNA	single-stranded DNA
T	thymine, or thymidine
τ	electric field pulse duration
TEMED	<i>N,N,N',N'</i> -tetramethylethylene diamine
TIR	terminal inverted repeat
TRF1	terminal recognition factor 1

tRNA	transfer RNA
TP	terminal protein
Tris	tris(hydroxymethyl)methylamine
U	uracil, or uridine
UCS	upstream conserved sequence
VPg	genome-linked viral protein
v/v	volume per volume
w/v	weight per volume
YRp	yeast replicating plasmid

CHAPTER 1

INTRODUCTION

1.1 LINEAR DNA PLASMIDS OF *KLUYVEROMYCES LACTIS*: ASSOCIATION WITH KILLER PHENOTYPE

Certain strains of the lactose-utilising budding yeast *Kluyveromyces lactis* secrete a protein toxin which inhibits the growth of sensitive yeasts (Gunge *et al.*, 1981). When grown against a background of sensitive cells on solid medium, this killer character causes a distinct halo of growth inhibition around colonies of the killer strain. A wide range of yeasts are sensitive to the killer toxin, including *Saccharomyces cerevisiae*, *S. italicus*, *S. rouxii*, *Kluyveromyces thermotolerans*, *Torulopsis glabrata*, *Candida utilis*, *C. intermedia*, and non-killer strains of *K. lactis*. The killing spectrum and mode of action of the *K. lactis* toxin are quite distinct from the dsRNA system of *S. cerevisiae* (Gunge and Sakaguchi, 1981).

Initial studies revealed that the killer phenotype was dependent upon the presence of two linear, dsDNA plasmids (Gunge *et al.*, 1981; Osami *et al.*, 1981) of 8.9 and 13.5 kb, which have been termed pGKL1 (or K1) and pGKL2 (or K2), respectively. As will be discussed, K1 and K2 possess terminal inverted repeat (TIR) sequences, and have proteins covalently linked to their 5' termini, the so-called terminal proteins (TPs). Structurally similar genetic elements are found in a variety of other systems, and have been termed invertrons (Sakaguchi, 1990).

1.2 LINEAR GENETIC ELEMENTS WITH TERMINAL INVERTED REPEAT SEQUENCES AND TERMINAL PROTEINS

Linear DNA molecules which have TIRs and proteins covalently bonded to their 5' ends have been found in bacteriophages, bacterial plasmids, yeast plasmids, fungal plasmids, plant mitochondrial plasmids, and animal viruses (Table 1.1). These linear DNA molecules range in length from the 1.9 kb *Fusarium oxysporum* mitochondrial plasmid, pFOXC2, to the 350 kb giant linear plasmid, pSCP1, from *Streptomyces coelicolor*. The length of the TIRs also show large variation. The *Bacillus subtilis* bacteriophage ϕ 29 has TIRs of only 6 bp, whereas the *S. coelicolor* giant linear plasmid, pSCP1, has TIRs of 81 kb. In some systems the size of the TP has been determined. The smallest TPs are found bonded to the

Table 1.1. Compilation of linear DNA elements with TIRs and TPs.

Organism	Plasmid	Size	TIR	TP	Localisation	Refs.
<u>Bacteriophages</u>						
<i>Bacillus subtilis</i> phage ϕ 29		19.3 kb	6 bp	28 kDa		†
<i>Escherichia coli</i> phage PRD1		14.5 kb	110-111 bp	28 kDa		†
<i>Streptococcus pneumoniae</i> phage Cp-1		18 kb	236 bp	28 kDa		1, 2
<u>Bacteria</u>						
<i>Streptomyces clavuligerus</i>	pSCL	12 kb	yes	yes		3, 4
<i>Streptomyces coelicolor</i>	pSCP1	350 kb	81 kb	yes		5-7
<i>Streptomyces rimosus</i>	pSRM	43 kb	nd	yes		8
<i>Streptomyces rochei</i>	pSLA1	17 kb	nd	yes		9, 10
<i>Streptomyces rochei</i>	pSLA2	17 kb	614 bp	yes		9, 10
<u>Animal viruses</u>						
Adenovirus		35-36 kb	103-163 bp	55 kDa		†
<u>Yeasts</u>						
<i>Pichia inositovora</i>	pPinl-1	18 kb	nd	yes	Cytoplasm	11, 12
<i>Pichia inositovora</i>	pPinl-2	13 kb	nd	yes	Cytoplasm	11, 12
<i>Pichia inositovora</i>	pPinl-3	10 kb	nd	yes	Cytoplasm	11, 12
<i>Saccharomycopsis crataegensis</i>	pScrl-1	15 kb	nd	yes	Cytoplasm	13, 14
<i>Saccharomycopsis crataegensis</i>	pScrl-2	7 kb	nd	yes	Cytoplasm	13, 14
<i>Saccharomycopsis crataegensis</i>	pScrl-3	5 kb	nd	yes	Cytoplasm	13, 14
<i>Saccharomyces kluyveri</i>	pSKL	14.2 kb	483 bp	yes	Cytoplasm	15, 16
<u>Fungi</u>						
<i>Ascobolus immersus</i>	pAI2	5.6 kb	~700 bp	yes	Mitochondria	17, 18
<i>Ceratocystis fimbriata</i>	pCF637	8.2 kb	nd	yes	Mitochondria	19
<i>Claviceps purpurea</i>	pC1K1	6.7 kb	327 bp	yes	Mitochondria	20
<i>Fusarium solani</i> f. sp. <i>cucurbitae</i>	pFSC1	9.2 kb	1211 bp	80 kDa	Mitochondria	21, 22
<i>Fusarium solani</i> f. sp. <i>cucurbitae</i>	pFSC2	8.3 kb	1027 bp	80 kDa	Mitochondria	21, 22
<i>Fusarium oxysporum</i>	pFOXC2	1.9 kb	50 bp	yes	Mitochondria	23, 24
<i>Lentinus edodes</i>	pLLE1	11 kb	nd	yes	Mitochondria	25
<i>Morchella conica</i>	pMC3-2	6 kb	710-713 bp	yes	Mitochondria	26, 27
<i>Neurospora crassa</i>	maranhar	7 kb	yes	yes	Mitochondria	28
<i>Neurospora intermedia</i>	kalilo	9 kb	1361 bp	120 kDa	Mitochondria	29-32
<i>Pleurotus ostreatus</i>	pLPO1	10 kb	nd	yes	Mitochondria	33
<i>Pleurotus ostreatus</i>	pLPO2	9.4 kb	nd	yes	Mitochondria	33
<i>Podospora anserina</i>	pAL2-1	8.4 kb	975 bp	yes	Mitochondria	34, 35

Continued on page 3

Table 1.1 (contd.)

Organism	Plasmid	Size	TIR	TP	Localisation	Refs.
Higher Plants						
<i>Brassica campestris/napus</i>	11.5 kb plasmid		325 bp	yes	Mitochondria	36
<i>Zea mays</i> N line	n	2.3 kb	170 bp	yes	Mitochondria	37
<i>Zea mays</i> S line	S-1	6.4 kb	208 bp	yes	Mitochondria	38-40
<i>Zea mays</i> S line	S-2	5.4 kb	208 bp	yes	Mitochondria	38-40

nd, not determined.

†, see text for references; 1, García *et al.*, 1983a; 2, Escarmís *et al.*, 1984; 3, Keen *et al.*, 1988; 4, Wu and Roy, 1993; 5, Kinashi and Shimajimurayama, 1991; 6, Kinashi *et al.*, 1992; 7, Kinashi *et al.*, 1991; 8, Chardon-Loriaux *et al.*, 1986; 9, Hirochika *et al.*, 1984; 10, Hirochika and Sakaguchi, 1982; 11, Hayman and Bolen, 1991; 12, Ligon *et al.*, 1989; 13, Bolen *et al.*, 1992; 14, Shepherd *et al.*, 1987; 15, Hishinuma and Keiko, 1991; 16, Kitada and Hishinuma, 1987; 17, Meinhardt *et al.*, 1986; 18, Francou, 1981; 19, Giasson and Lalonde, 1987; 20, Düvell *et al.*, 1988; 21, Samac and Leong, 1989; 22, Samac and Leong, 1988; 23, Meinhardt *et al.*, 1990; 24, Kistler and Leong, 1986; 25, Katayose *et al.*, 1990; 26, Rohe *et al.*, 1991; 27, Meinhardt and Esser, 1987; 28, Court *et al.*, 1991; 29, Chan *et al.*, 1991; 30, Vierula *et al.*, 1990; 31, Bertrand and Griffiths, 1989; 32, Bertrand *et al.*, 1986; 33, Yui *et al.*, 1988; 34, Hermanns and Osiewacz, 1992; 35, Osiewacz *et al.*, 1989; 36, Turpen *et al.*, 1987; 37, Leon *et al.*, 1989; 38, Paillard *et al.*, 1985; 39, Levings and Sederoff, 1983; 40, Kemble and Thompson, 1982.

B. subtilis phage ϕ 29, the *Escherichia coli* bacteriophage PRD1, and the *Streptococcus pneumoniae* bacteriophage Cp-1 invertrons, all having a M_r of 28 kDa. The largest found, to date, is the 120 kDa TP of the *kalilo* plasmid from *Neurospora intermedia*.

Although many linear plasmids have been described in various organisms, very little is known about their function. Where phenotypes have been assigned, they have been shown to be as diverse as the range of organisms in which they have been found. The bacterial giant linear plasmid, pSCP1, codes for the methylenomycin biosynthetic genes in *S. coelicolor*. The yeast *Pichia inositovora* contains three linear plasmids, pPinl-1, pPinl-2, and pPinl-3, which are associated with a novel killer toxin activity. Among all fungal mitochondrial plasmids examined so far, few elements have been correlated with a function. The integration of the *Neurospora* plasmids, *kalilo* and *maranhar*, into the mitochondrial genome appears to be causatively involved in the control of senescence. However, in contrast to this situation, the longevity of the *Podospora* mutant AL2 has been correlated with the presence and integration of a linear DNA species, termed pAL2-1, into the mtDNA. Plasmids S-1 and S-2 are associated with maternally inherited male sterility, and can recombine with, and cause structural changes in, the mitochondrial genome of *Zea mays*. However, the consequences of the maize mtDNA rearrangements are not known.

Emerging sequence data have been used to unravel the evolutionary relationships of the linear plasmids (Kempken *et al.*, 1992). Evolutionary trees deduced from both DNA and RNA polymerases show a clear separation of yeast cytoplasmic plasmids from mitochondrial localised plasmids. This is most probably a result of adaptation to different intracellular environments.

In their structural and genetic characteristics, linear plasmids resemble viral genomes. Two models have been proposed to explain how linear plasmids first entered cells. On the one hand, linear plasmids might be derived from linear DNA viruses that originated as parts of bacteria (Meinhardt *et al.*, 1990). It is generally accepted that mitochondria are remnants of endosymbiotic bacteria. In parallel, the bacterial viruses may have lost several functions (e.g., functions for capsid formation) giving rise to today's linear plasmids. This idea is supported by several lines of evidence: 1, the structural similarity to bacteriophages ϕ 29 and PRD1; 2, results indicating that these viruses share significant sequence similarity to mitochondrial linear plasmids (Kempken *et al.*, 1992); and 3, the occurrence of linear plasmids which resemble noninfectious bacteriophages in *Streptomyces clavuligerus* (Keen *et al.*, 1988). Similarly, yeast linear plasmids may have evolved from cytoplasmic viruses.

Alternatively, a virus may have infected mitochondria or yeast cells and become trapped inside the organelle or cell (Kempken *et al.*, 1992). Subsequently, the virus lost the capsid encoding genes. In support of this hypothesis, the infection of organelles by the tobacco mosaic virus (TMV) has been demonstrated (Schoelz and Zaitlin, 1989). However, TMV is a RNA virus without the typical characteristics of linear plasmids.

The best understood invertrons are provided by adenovirus and the *B. subtilis* bacteriophage $\phi 29$. In both systems the TP has been shown to provide the primer for DNA replication. It is likely that those linear replicons that contain a TP also replicate their DNA by this so-called protein-priming mechanism. A TP is also a characteristic of the hepadnavirus family (Weiser *et al.*, 1983; Molnar-Kimber *et al.*, 1983; Gerlich and Robinson 1980), and certain groups of animal and plant RNA viruses have been shown to contain a genome-linked viral protein (VPg) (Vartapetian and Bogdanov, 1987; Daubert and Bruening, 1984; Wimmer, 1982). In some of these systems, the covalently linked protein plays a similar role to that of the TP of invertrons.

1.3 REPLICATION OF LINEAR DNA MOLECULES

For a review of DNA replication see Kornberg and Baker (1992).

1.3.1 General Mechanism of DNA Replication

DNA polymerases, catalysts for DNA chain growth, have been found in extracts of all bacterial, plant, and animal cells where DNA synthesis has been observed. Biosynthesis of DNA has three basic requirements. Firstly, all four of the activated precursors (the deoxyribonucleoside 5'-triphosphates, dATP, dCTP, dGTP, and dTTP) must be present. Secondly, as all known DNA polymerases add dNTPs to the 3'-OH terminus of a pre-existing DNA or RNA chain, a primer chain with a free 3'-OH group is required. Thirdly, a DNA template is essential because DNA polymerase is a template-directed enzyme.

The chain elongation reaction proceeds by nucleophilic attack of the 3'-OH terminus of the primer. A phosphodiester bridge forms between the 3'-OH of the primer and the 5'-phosphate group of the incoming dNTP, and pyrophosphate is concomitantly released. The subsequent hydrolysis of pyrophosphate by inorganic pyrophosphatase, a ubiquitous enzyme, drives the polymerisation reaction forward. Elongation of the DNA chain thus proceeds in the 5' to 3' direction.

1.3.2 The Problem Confronting Linear Replicons

No known DNA polymerase is able to initiate synthesis *de novo*, a free 3'-OH group is required. Thus, an additional mechanism is required to provide the synthesis primer. In many cases this is provided by RNA polymerase which can start chains *de novo*, and the first dNTP residue to be inserted is added to the terminal 3'-OH of this RNA strand.

This mechanism can account for daughter strand DNA synthesis on a circular DNA molecule, or linear DNAs that circularise or form concatemers (see Section 1.3.3). However, the fact that none of the known DNA polymerases are able to initiate DNA chains, or synthesise in the 3' to 5' direction, raises a problem for linear replicons since there is no way of filling the gap resulting at the 5' ends of the newly synthesized DNA chain after the removal of the RNA primer.

The minimum length requirement of an RNA primer is two ribonucleotides (B. Wilkins, personal communication). Therefore, resulting daughter strands would be two dNTPs shorter than the parental strands after each round of DNA replication. Furthermore, it is not known whether RNA terminal priming is feasible. We usually think of a polymerase as binding to the DNA template at a site surrounding the position at which a base is to be incorporated.

Linear replicons face a further problem as they are susceptible to degradation by exonucleases present within the cell. Clearly, the termini of linear DNA molecules must be modified in some way to maintain their integrity and to permit the replication of full-length progeny DNA molecules.

1.3.3 Solutions for the Replication of the Ends of Linear DNA Molecules

As already mentioned, there is no problem for linear replicons to copy a terminus if they are able to form circular or concatemeric molecules. The genome of the *E. coli* bacteriophage λ (Hendrix *et al.*, 1983; Black, 1988) has a 12 nucleotide single-stranded terminus at each 5' end. These termini are complementary and therefore cohesive. After the DNA enters the *E. coli* cell, cohesion of the complementary 5' ssDNA ends converts the linear duplex to a circular form. Covalent closure of the opposed ends by DNA ligase and negative supercoiling by DNA gyrase prepare the DNA for transcription, replication, recombination, or integration. Early and late stages of replication are characterised by differently structured intermediates. Circular forms, produced by bidirectional replication, predominate early after the virus initiates replication, and homologous recombination yields multimeric circles. Long concatemeric forms, produced by rolling-circle replication,

predominate late in a productive infection. Multimeric λ DNA, essential for packaging into phage particles, is cleaved by the λ terminase precisely at the cohesive-end sites to produce unit-length linear duplexes with complementary 5' ssDNA ends.

The genome of the *E. coli* bacteriophage T7 has a stretch of several hundred nucleotides repeated at each end (Watson, 1972). Limited removal of 3' terminal regions by exonuclease III leaves 5' cohesive tails that link intramolecularly to form circles or, at higher DNA concentrations, link intermolecularly to form concatemers. Gap-filling by polymerase and sealing by ligase ensure that the concatemer is covalently intact. Unit-length genomes are produced for packaging into virions by specific staggered endonuclease nicking of both strands whose ends can then be filled out by polymerase.

In some cases, linear DNA molecules contain a palindromic nucleotide sequence at their 3' ends that allows the formation of a hairpin structure which provides the needed 3'-OH group for initiation and elongation, and the replication of the 5' termini by a hairpin-loop transfer mechanism. This mechanism, first proposed by Cavalier-Smith (1974) for eukaryotic DNA replication, has been shown to take place in parvoviruses (see Section 1.4.10).

All linear eukaryotic chromosomes examined to date exhibit a terminal structure known as a telomere, which consists of stretches of very simple, tandemly repeated sequences. Although the simple telomeric repeats do not conform to a specific consensus sequence between species, they have a G-rich strand with an orientation specificity with respect to the end of the chromosome. At each chromosomal end, the G-rich telomeric strand runs 5' to 3' towards the terminus, and protrudes 12-16 nucleotides beyond the complementary C-rich strand in the various species in which sequence analysis has been possible (Klobutcher *et al.*, 1981; Henderson and Blackburn, 1989).

The G-rich strand of telomeres is synthesised by an unusual ribonucleoprotein reverse transcriptase, telomerase, where the associated RNA is an intrinsic part of the enzyme. Telomerase activities have been detected *in vitro* in ciliate (Shippen-Lentz and Blackburn, 1990; Shippen-Lentz and Blackburn, 1989; Greider and Blackburn, 1989; Zahler and Prescott, 1988; Greider and Blackburn, 1987; Greider and Blackburn, 1985) and human (Morin, 1989) cell-free extracts. The telomerase RNAs of *Tetrahymena* and *Euplotes* have been identified, and studies *in vitro* indicate that the RNA acts as the template for synthesis of the G-rich telomeric DNA strand (Shippen-Lentz and Blackburn, 1990; Greider and Blackburn, 1989).

Evidence that telomerase activity is essential for long-term viability comes from analysis of telomerase RNA mutations *in vivo*. Overexpression of one particular mutant telomerase

RNA gene in *Tetrahymena* is sufficient to cause a dominant negative phenotype characterised by telomere shortening and senescence (Yu *et al.*, 1990). In yeasts, inactivation of the gene *EST1*, known to be required for long-term viability, causes steady and continuous telomere shortening over several cell generations and eventual senescence (Lundblad and Szostak, 1989). Cell death is preceded by increased rates of chromosome loss. The *EST1* gene encodes a reverse transcriptase-like protein (Lundblad and Blackburn, 1990). This, together with the identification of the ciliate telomerases as specialised reverse transcriptases (Shippen-Lentz and Blackburn, 1990; Yu *et al.*, 1990; Greider and Blackburn, 1989), and the phenotype of *est1* deficient mutants, indicates that EST1 is a protein component of telomerase (Lundblad and Blackburn, 1990). These findings imply that telomerase is essential for maintenance of telomere length and long-term viability in yeasts as well as *Tetrahymena*.

The structure for the 3' overhang of the *Oxytricha* telomere has been determined by crystallography (Kang *et al.*, 1992) and NMR (Smith and Feigon, 1992). The structure demonstrates that two *Oxytricha* G-rich strands can associate to form a G-quadruplex as a structural motif. This structure could be the basis for a linear assembly of the DNA fragments in the *Oxytricha* macronucleus, and may protect termini from recognition by exonucleases.

For many linear replicons, copying of a terminus cannot take place by any of the above mechanisms. In such cases a protein is used to make a priming reaction possible. Invertrons use this mechanism to replicate their DNA.

1.4 PROTEIN-PRIMING OF DNA REPLICATION

The primer terminus required for DNA replication can be provided by a OH group of an amino acid within a protein, rather than by a RNA or DNA molecule. Serine, threonine, and tyrosine bear a free β -OH group, and all have been found capable of forming a covalent linkage to the nucleic acid. The DNA polymerase covalently links the initiating base-paired dNTP to an amino acid side chain of the terminal protein, and then uses this nucleotide to prime the extension of the DNA chain. The TP, or a processed form of it, remains linked to the 5' ends of the genome. A single priming event for each of the two strands of a linear genome should suffice for replication of the templates, and no discontinuous replication need be involved.

The adenoviruses of animal cells, certain *B. subtilis* phages and the *E. coli* phage PRD1 are the best-studied examples of protein-primed replication among invertrons. Other invertrons possessing a TP are also likely to replicate their genomes by protein-priming. A TP bound

to the 5' end of the duck hepatitis B virus genome is believed to be involved in a specialised protein-priming mechanism for reverse transcription. In addition, certain TP-containing RNA viruses may utilise this mechanism to replicate their genomes.

1.4.1 Structure of Genome Termini

The Adenoviruses (Ginsberg, 1984), which transform cells and produce cancer in certain animals, are icosahedrons, about 80 nm in diameter, made up of 252 capsomers. Of these, 240 hexons each face six neighbours, and 12 pentons, located at each of the 12 vertices, each face five neighbours. Projecting from each penton is a fibre with a knob at the end through which the virus is adsorbed to cell surface receptors and penetrates the plasma membrane. The nucleocapsid is formed from DNA and four polypeptides, one of which is the adenovirus TP. Despite the absence of histones, the DNA is organised in a chromatin-like structure.

The human adenovirus genome consists of a linear dsDNA of 36 kb with a covalently bound 55 kDa TP (Rekosh *et al.*, 1977; Carusi, 1977) linked to the 5' terminus of each strand, and TIRs of between 103 and 163 bp depending upon the adenovirus serotype (Shinagawa and Padmanabhan, 1980). The presence of a TP was first suggested by the finding of circular molecules and concatemers in the DNA isolated from viral particles, and their conversion into linear, unit-length DNA after protease treatment (Robinson *et al.*, 1973). In addition, the infectivity of the adenovirus 5 DNA-protein complex was greatly reduced after treatment with pronase (Sharp *et al.*, 1976). A model first proposed by Rekosh *et al.* (1977) suggested that a free molecule of the TP could act as a primer for the initiation of replication. The TP is derived from a 80 kDa precursor protein (pTP) by cleavage late in the infective cycle (Binger *et al.*, 1982; Challberg and Kelly, 1981; Stillman *et al.*, 1981; Challberg *et al.*, 1980). The linkage between both the pTP and the TP and DNA, readily hydrolysed in alkali, is a phosphodiester bond between the β -OH group of a serine residue at position 580 of the pTP and 5' dCMP (Smart and Stillman, 1982; Desiderio and Kelly, 1981; Challberg *et al.*, 1980).

The *Bacillus subtilis* phage ϕ 29 genome consists of a linear dsDNA of 19,285 bp (Vlcek and Paces, 1986; Garvey *et al.*, 1985; Yoshikawa and Ito, 1982) with a covalently bound 28 kDa TP (Ito, 1978; Salas *et al.*, 1978; Yehle, 1978), and TIRs of 6 bp (Escarmís and Salas, 1981). The presence of a TP was first suggested by the finding that ϕ 29 DNA could be isolated from phage particles as circular and concatemeric forms, and that these DNA structures could be converted into unit-length linear molecules by treatment with proteolytic enzymes (Ortín *et al.*, 1971). In addition, the transfection of ϕ 29 DNA was sensitive to protease treatment (Hirokawa, 1972), and the DNA isolated from a ϕ 29 *ts*

mutant in gene 3 was thermolabile for transfection (Yanofsky *et al.*, 1976). Subsequently, it was shown that a protein, characterised as the product of the viral gene 3, was covalently linked at the 5' termini of $\phi 29$ DNA (Salas *et al.*, 1978).

Other *B. subtilis* phages morphologically similar to $\phi 29$, such as $\phi 15$, $\phi 21$, PZA, PZE, Nf, M2, B103, and GA-1, have also been found to contain linear dsDNA, and TPs of similar size (Gutiérrez *et al.*, 1986a; Geiduschek and Ito, 1982; Yoshikawa and Ito, 1981; Fucik *et al.*, 1980). All these phages have a short TIR; six nucleotides long, with the consensus sequence AAAGTA, for $\phi 29$, $\phi 15$, PZA, and B103 DNAs; seven nucleotides long, with the consensus sequence AAATAGA, for GA-1 DNA; and eight nucleotides long, with the consensus sequence AAAGTAAG, for Nf and M2 DNAs. The amino acid sequence of the TP of phage PZA (Paces *et al.*, 1985) is almost identical to that of phage $\phi 29$ (Escarmís and Salas, 1982; Yoshikawa and Ito, 1982), and the amino acid sequence of the TP of phage Nf is approximately 66% similar with the $\phi 29$ and PZA TPs, showing similar hydropathy and secondary structure predictions (Leavitt and Ito, 1987). The $\phi 29$ TP can functionally substitute the TP of phage PZA *in vivo*, but replication of phage Nf DNA cannot take place *in vivo* using the $\phi 29$ TP as a primer (Bravo *et al.*, 1994). The linkage between the $\phi 29$ TP and DNA, readily hydrolysed in alkali, is a phosphodiester bond between the β -OH group of a specific serine residue at position 232 in the 266 amino acid TP and 5' dAMP (Hermoso *et al.*, 1985; Hermoso and Salas, 1980).

Bacteriophage PRD1 is a prototype of small lipid-containing phages that infect a wide variety of Gram-negative bacteria, ranging from *E. coli* and *Salmonella typhimurium* to *Pseudomonas aeruginosa* (Mindich and Bamford, 1988). The genome of PRD1 is a linear, dsDNA of about 14.7 kb, with a 28 kDa TP covalently linked to the 5' termini of the viral genome (Bamford *et al.*, 1983). The linkage between the TP and PRD1 DNA is a phosphodiester bond between a tyrosine residue, at position 190 of the 259 amino acid TP, and dGMP (Shiue *et al.*, 1991; Bamford and Mindich, 1984). The PRD1 DNA contains TIR sequences of 110 bp (Savilahti and Bamford, 1986).

1.4.2 Electron Microscopic Analysis

Electron microscopic and restriction enzyme analysis of viral intermediates from adenovirus-infected nuclei (Sussenbach and Kuijk, 1978; Sussenbach and Kuijk, 1977, Sussenbach *et al.*, 1972), or cells (Lechner and Kelly, 1977), indicated that initiation of replication occurs at both ends of the DNA molecule, and proceeds by a strand-displacement mechanism giving rise to two basic types of replicating molecules (Figure 1.1). Type I molecules consisted of unit-length linear duplexes with one or more single-

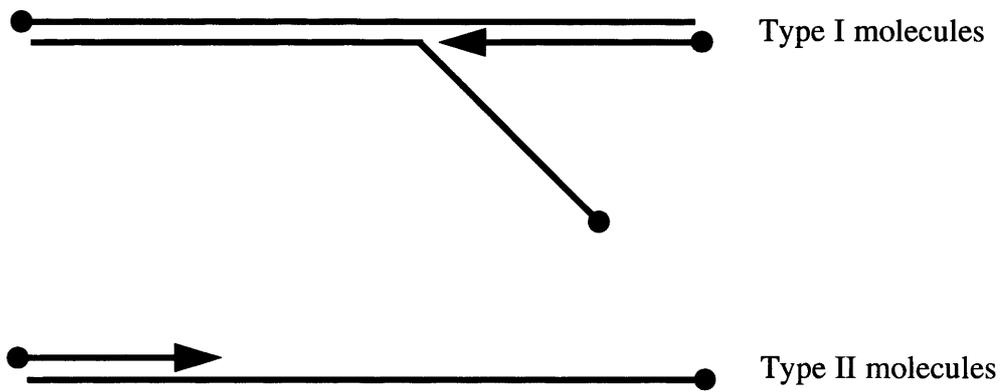


Figure 1.1. DNA replication intermediates of adenovirus and $\phi 29$ visualised by electron microscopic analysis. ● represents 5'-TPs. Arrowheads indicate direction of replication. See Sections 1.4.2 and 1.4.6 for details.

stranded branches from the same or different DNA end, and accounted for about 33% of the molecules (Lechner and Kelly, 1977). Type II forms consisted of unit-length linear molecules that were partially single-stranded, and accounted for about 54% of the molecules (Lechner and Kelly, 1977). These replicative forms are best interpreted by knowledge of the *in vitro* replication mechanism (see Sections 1.4.3 and 1.4.6).

Electron microscopic analysis of replicative intermediates isolated from ϕ 29-infected *B. subtilis* showed the presence of two basic types of replicating DNA molecules (Sogo *et al.*, 1982; Harding and Ito, 1980; Inciarte *et al.*, 1980), similar to the structures found in adenovirus-infected cells. Type I and Type II molecules accounted for about 20% and 50%, respectively, of the total number of DNA molecules (Inciarte *et al.*, 1980). Type I molecules with a single-stranded branch from each DNA end were also seen. The results indicate that replication starts at either end of the DNA, and proceeds by a strand displacement mechanism (see Section 1.4.6).

1.4.3 In Vitro Replication Systems

The development of an *in vitro* DNA replication system, consisting of soluble extracts from the nuclei of HeLa cells infected with adenovirus 5, using as template DNA-protein complex isolated from adenovirus 5 virions (Stillman, 1983; Challberg and Kelly, 1979a; Challberg and Kelly, 1979b), defined three essential viral proteins, and three cellular proteins needed for efficient replication. Two of the virus-encoded proteins were required for the initiation of adenovirus DNA replication, the 80 kDa pTP and a 140 kDa DNA polymerase (Ad pol). The genes coding for these two replication proteins map in the E2B region that encodes an mRNA that allows the expression of both proteins after splicing (Stillman *et al.*, 1981). The pTP and Ad pol were co-purified in a functional form from adenovirus 2-infected HeLa cells (Ostrove *et al.*, 1983; Stillman *et al.*, 1982; Enomoto *et al.*, 1981). The pTP-Ad pol complex was separated by sedimentation in glycerol gradients containing urea, and reconstitution experiments indicated that both proteins are required for initiation activity (Friefeld *et al.*, 1983, Lichy *et al.*, 1982; Stillman *et al.*, 1982).

The third viral protein required for adenovirus DNA replication was the DNA binding protein (DBP), an early protein synthesised in large amounts in adenovirus-infected cells (van der Vliet and Levine, 1973). The protein contains 529 amino acids (Kruijer *et al.*, 1981), and binds cooperatively to single-stranded DNA, although binding to dsDNA has also been reported (Ariga *et al.*, 1980; Kaplan *et al.*, 1979; van der Vliet *et al.*, 1978; van der Vliet and Levine, 1973). DBP was essential for *in vitro* adenovirus DNA replication with purified proteins (Nagata *et al.*, 1983a; Ikeda *et al.*, 1981) and, although not absolutely required for initiation, stimulated pTP-dCMP complex formation (de Vries *et*

al., 1985; Nagata *et al.*, 1982). The study of the elongation reaction catalysed by the adenovirus DNA polymerase using poly(dT)-oligo(dA) as template-primer showed that the presence of DBP greatly increased the processivity of the polymerase and allowed its translocation through duplex DNA (Field *et al.*, 1984).

Genes 1, 2, 3, 5, 6, and 17 of ϕ 29 have been shown to be required for the synthesis of the viral DNA *in vivo* (Prieto *et al.*, 1989; Carrascosa *et al.*, 1976; Hagen *et al.*, 1976; Talavera *et al.*, 1972). A ^{32}P -labelled protein with the electrophoretic mobility of the gene 3 protein, p3, was found when extracts from ϕ 29-infected *B. subtilis* were incubated with [α - ^{32}P] dATP in the presence of ϕ 29 DNA-TP complex as template (Shih *et al.*, 1984; Peñalva and Salas, 1982; Shih *et al.*, 1982; Watabe *et al.*, 1982). Incubation of the ^{32}P -labelled protein with piperidine, under conditions in which the ϕ 29 DNA-TP linkage is hydrolysed, released 5' dAMP, indicating the formation of a TP-dAMP covalent complex (Peñalva and Salas, 1982).

Genes 2 and 3 were shown to be essential for the *in vitro* initiation reaction (Blanco *et al.*, 1983). Both genes have been cloned (Blanco *et al.*, 1984; Watabe *et al.*, 1984; García *et al.*, 1983b), and the proteins overproduced and highly purified in a functional form (Blanco and Salas, 1985; Blanco and Salas, 1984; Prieto *et al.*, 1984). The purified p2 protein, in addition to catalysing the initiation reaction, was shown to have DNA polymerase activity (Blanco and Salas, 1984; Watabe *et al.*, 1984). The enzyme consists of a single polypeptide of 66.5 kDa, and is able to catalyse the formation of the initiation complex between the TP and dAMP. The *in vitro* initiation reaction is greatly stimulated by NH_4^+ ions (Blanco *et al.*, 1987) because the ions stabilise the formation of the DNA-TP complex (Watabe *et al.*, 1984). In fact, the two proteins, p2 and p3, co-purify from extracts of ϕ 29-infected cells (Matsumoto *et al.*, 1984; Watabe *et al.*, 1983).

Analysis of ϕ 29 DNA polymerase activity in various *in vitro* DNA replication systems indicates that it is the only enzyme required for efficient synthesis of full length ϕ 29 DNA, with the TP, the initiation primer, as the only additional protein requirement (Blanco *et al.*, 1989; Blanco and Salas, 1985). Using primed M13 DNA as template, the ϕ 29 DNA polymerase is able to synthesise DNA chains greater than 70 kb. These results indicate that the ϕ 29 DNA polymerase is highly processive, and is able to produce strand displacement without the need of accessory proteins (Blanco *et al.*, 1989). By using appropriate amounts of TP, DNA polymerase, and proteins p5 and p6, the ϕ 29 DNA molecule was amplified by three orders of magnitude after one hour of incubation at 30°C (Blanco *et al.*, 1994). Moreover, the infectivity of the amplified material was demonstrated to be identical to that of the natural ϕ 29 DNA obtained from virions. These results establish the basic requirements for the development of isothermal DNA amplification strategies, based on

the ϕ 29 DNA replication machinery, that would be adequate for faithful amplification of DNA molecules longer than 70 kb.

The purified p5 protein has been shown to bind single-stranded DNA, and greatly stimulates ϕ 29 DNA replication at incubation times where the replication in the absence of p5 levelled off (Martín *et al.*, 1989). The p6 protein stimulates the formation of the TP-DNA initiation complex (Blanco *et al.*, 1986; Pastrana *et al.*, 1985), and also stimulates the transition from initiation to elongation (Blanco *et al.*, 1988). It binds specifically to the ends of the viral DNA and produces a conformational change in the DNA that probably induces the unwinding of the double helix to expose a region of ssDNA (Serrano *et al.*, 1990). The role of the products of genes *1* and *17* has not yet been elucidated.

The left-hand terminus of the PRD1 genome codes for two very early proteins, the TP (gene *VIII*, protein p8) and a 553 amino acid DNA polymerase (gene *I*, protein p1), in this order from the genome terminus (Hsieh *et al.*, 1987; Jung *et al.*, 1987a; Savilahti and Bamford, 1987; Bamford and Mindich, 1984; Bamford *et al.*, 1983; McGraw *et al.*, 1983; Mindich *et al.*, 1982). A DNA replication system with cell extracts has indicated that replication can start at either end of the molecule, and that the TP and DNA polymerase are the only phage-encoded proteins needed to synthesise the full-length phage genome *in vitro* (Yoo and Ito, 1989). Using purified components of the PRD1 replication system, the participation of host factors in replication *in vitro* was ruled out (Savilahti *et al.*, 1991).

1.4.4 DNA Sequence Requirements

The identical origins at each end of the adenovirus genome consist of domains A, B, and C. Domain A (nucleotides 1-18) is essential and can serve as a minimal origin (Wides *et al.*, 1987; Guggenheimer *et al.*, 1984; Lally *et al.*, 1984; Rawlins *et al.*, 1984; Tamanoi and Stillman, 1983). It is very AT-rich and contains the sequence 9-ATAATATACC-18, fully conserved in different adenovirus serotypes (Challberg and Rawlins, 1984; Shinagawa *et al.*, 1983; Aleström *et al.*, 1982; Tolun *et al.*, 1979). Mutations in this conserved sequence greatly reduce the template activity, whereas several base pairs in region 1-9 can be mutated without loss of activity (Challberg and Rawlins, 1984; Tamanoi and Stillman, 1983). The 9-18 conserved region may be involved in the binding of the pTP (Rijnders *et al.*, 1983). Deletion of domain B (nucleotides 19-39), with the consensus sequence TTGGCN₅GCCAA, decreases the efficiency of initiation approximately tenfold (Wides *et al.*, 1987; de Vries *et al.*, 1985; Guggenheimer *et al.*, 1984; Rawlins *et al.*, 1984). Deletion of domain C (nucleotides 39-51), with the consensus sequence TATGATAAT, decreases

the efficiency of the initiation reaction about threefold (Wides *et al.*, 1987; Pruijn *et al.*, 1986; van der Vliet *et al.*, 1978).

The DNA sequence requirements for the ϕ 29 initiation reaction have been studied by cloning ϕ 29 DNA fragments from the left and right ends (Gutiérrez *et al.*, 1986b). Fragments released by restriction nuclease digestion, containing the ϕ 29 DNA TIRs, were active templates for the initiation reaction. No template activity was obtained with the circular plasmid, or when the TIR sequences were not placed at the DNA ends. The analysis of deletion derivatives indicates that the minimal origins of replication are located within the terminal 12 bp at each ϕ 29 DNA end (Gutiérrez *et al.*, 1988). Site-directed and random mutagenesis in those sequences indicates that a change of the second or third A in the ϕ 29 DNA sequence into a C completely abolishes template activity. Changes at positions 4-12 were tolerated to a great extent, suggesting that the sequence requirements at the proximal region of the replication origin are more strict than those at the distal region (Gutiérrez *et al.*, 1988).

1.4.5 Participation of Host Proteins

Three host proteins are required for efficient Adenovirus replication. These are two transcriptional activators, nuclear factor 1 (NF1) and nuclear factor III (NFIII), and a topoisomerase (NFII). NF1 binds specifically to domain B (Rosenfeld *et al.*, 1987; de Vries *et al.*, 1987; Diffley and Stillman, 1986; Rosenfeld and Kelly, 1986; Leegwater *et al.*, 1985; de Vries *et al.*, 1985, Nagata *et al.*, 1983b), stimulates the initiation reaction, and becomes essential in the presence of DBP (de Vries *et al.*, 1985; Nagata *et al.*, 1982). NF1 has been purified (Jones *et al.*, 1987; Rosenfeld *et al.*, 1987; Diffley and Stillman, 1986), and shown to be indistinguishable from the transcription factor CTF (cellular transcription factor) (Jones *et al.*, 1987). The amino-terminal 220 amino acids of NF1 are sufficient for specific DNA binding, protein dimerisation, and adenovirus replication, whereas transcriptional activation requires an additional carboxy-terminal domain (Gounari *et al.*, 1990; Mermod *et al.*, 1989). In the presence of DBP, the affinity of NF1 for its binding site is increased (Cleat and Hay, 1989). This is due to the formation of a multimeric protein complex of DBP and dsDNA that enhances the binding of NF1 (Stuiver and van der Vliet, 1990). The NF1 site can be inverted or replaced by cellular NF1 binding sites, but its position must be fixed. Insertion or deletion of 1 or 2 bp between the minimal origin and domain B abolishes the stimulatory effect of this domain (Wides *et al.*, 1987; de Vries *et al.*, 1987; Adhya *et al.*, 1986). This critical spacing requirement suggests the existence of specific interactions between the factors that recognise domains A and B (Kelly and Wold, 1988).

NFII is required for the elongation of replicating intermediates of adenovirus DNA to full-length, and was isolated from uninfected HeLa cells (Nagata *et al.*, 1983b). NFIII stimulates the initiation of adenovirus DNA replication *in vitro* and binds to domain C (O'Neill and Kelly, 1988; Pruijn *et al.*, 1987; Pruijn *et al.*, 1986; van der Vliet *et al.*, 1978). The sequences recognised by NFIII are similar to promoter and enhancer elements (Prujn *et al.*, 1989; O'Neill and Kelly, 1988; Pruijn *et al.*, 1987). Indeed, NFIII is identical to the octamer transcription factor-1 (OTF-1) (Prujn *et al.*, 1989; O'Neill *et al.*, 1988). NFIII has a conserved DNA binding domain (POU domain) present in several transcription factors, and this 160 amino acid domain is sufficient for the stimulation of adenovirus DNA replication *in vitro* (Verrijzer *et al.*, 1990). Stimulation by the POU domain is caused by an interaction with the viral pTP-Ad pol complex (Coenjaerts *et al.*, 1994).

1.4.6 Mechanism of DNA Replication

The adenovirus type 5 origin starts with the sequence 3' GTAGTA. By employing a reconstituted replication system, with both synthetic oligonucleotides and the natural TP-DNA as templates, it was shown that pTP-CAT, rather than pTP-C, was the intermediate in initiation (King *et al.*, 1994). Furthermore, by replicating oligonucleotide templates mutated at different positions and analysing the product lengths, it was also observed that the sequence GTA at positions 4-6, rather than 1-3, was used as a template for pTP-CAT formation. Moreover, deletions of one or two nucleotides at the molecular ends were regenerated upon *in vitro* replication. These results support a model in which the pTP-CAT intermediate, synthesised opposite to positions 4-6, jumps back to position 1 of the template to start elongation. It has been proposed that this jumping-back mechanism ensures the integrity of terminal sequences during replication of the linear genome (King *et al.*, 1994). This model is similar to the sliding back models proposed for ϕ 29 and PRD1 DNA replication (see below).

For the replication of the displaced strand, two models have been proposed. In one, complete displacement of the parental strand is assumed to occur with the formation of a panhandle structure by hybridisation of the self-complementary terminal sequences. The duplex end of the panhandle has the same structure of the duplex viral genome and is probably recognised, initiated, and replicated in the same way. Intermediates in this stage appear as partially duplex molecules (Type II forms). In the other, before displacement synthesis initiated at one end of the DNA is completed, initiation occurs at the other end, giving rise to Type I molecules with two single-stranded tails. When the two replication forks meet, separation occurs, producing Type II molecules.

Panhandle molecules have not been found *in vivo*. However, an artificial panhandle molecule containing the adenovirus 2 TIR was shown to be an efficient template for initiation of replication by protein-priming (Leegwater *et al.*, 1988). Also, adenoviruses with non-identical terminal sequences are viable, giving rise after transfection to DNA molecules with identical termini. These results could be explained by strand displacement with the formation of a panhandle structure and efficient repair of one end, with the opposite terminus used as template (Lippé and Graham, 1989). This finding was taken as evidence for the existence of the panhandle structure as a replicative intermediate *in vivo*. However, the possibility that *in vivo* complementary strand synthesis occurs from both ends of adenovirus DNA molecules cannot be excluded if it is taken into account that such replicating molecules have been found (Lechner and Kelly, 1977).

Processing of pTP to TP is not required for continued adenovirus DNA replication and is accomplished during virion assembly (Binger *et al.*, 1982; Challberg and Kelly, 1981; Stillman *et al.*, 1981). The fate of the amino-terminal fragment is not clear. Mutations near the amino-terminus of pTP block its activity *in vitro* (Pettit *et al.*, 1988), and affect its function *in vivo* (Freimuth and Ginsberg, 1986). Using vaccinia virus-expressed protein, pTP has been shown to interact with the nuclear matrix *in vivo* and *in vitro* (Fredman and Engler, 1993). Furthermore, reduced nuclear matrix association of viral DNAs with pTP containing mutations in the amino-terminal portion, suggests that the amino-terminal fragment remains associated with the carboxy-terminal 55 kDa fragment, and contains the nuclear matrix binding site (Schaak *et al.*, 1990). Alternatively, these mutations could exert their phenotype through partial denaturation of the 55 kDa TP portion of pTP. The association of pTP with matrix is postulated to help direct adenovirus replication complexes to the appropriate location within the nucleus (Fredman and Engler, 1993).

Using synthetic oligonucleotides, a mutational analysis of the ϕ 29 DNA right end indicated that initiation of replication occurs opposite the second nucleotide at the 3' end of the template, that a terminal repetition of at least two nucleotides is required to efficiently elongate the initiation complex, and that all the nucleotides of the template, including the 3' terminal one, are replicated (Méndez *et al.*, 1992). These results support a mechanism where the TP-dAMP initiation complex slides backwards locating dAMP opposite the first T of the template. This sliding-back mechanism for ϕ 29 DNA replication differs to that proposed for adenovirus in that the adenovirus intermediate contains a trinucleotide rather than just one dNMP. This makes it necessary to propose a jumping-back transition, rather than a sliding-back transition, for adenovirus DNA replication, to prevent mismatches that might occur during a sliding back process.

The initiation of ϕ 29 and adenovirus DNA replication takes place at either end of the DNA, and proceeds by strand displacement (Inciarte *et al.*, 1980; Lechner and Kelly,

1977). However, the initiation mechanism of the parental strand which is being displaced is still unknown. In the adenovirus system it has been suggested that DNA elongation by strand displacement produces a double-stranded molecule and a displaced single-stranded molecule (Hay and McDougall, 1986). However, there is no evidence for such a displacement, and initiation of replication before the strand has been completely displaced has been proposed as an alternative model for adenovirus and ϕ 29 DNA replication, taking into account the finding of *in vivo* replicative intermediates which consist of dsDNA with two single-stranded tails (Inciarte *et al.*, 1980; Lechner and Kelly, 1977). This mechanism has the advantage of allowing the proteins involved in the initiation of replication to recognise the same structure in the replication of both DNA strands. This could, of course, also be the case if the displaced single-stranded molecule could form a panhandle structure due to the presence of long TIRs. However, the very short TIRs present in ϕ 29 DNA (6 bp) make the formation of this structure unlikely.

To test the *in vivo* replication of the nontemplate DNA strand, recombinant ϕ 29 DNA molecules containing parental TP at only one end were constructed (Escarmís *et al.*, 1989). No replication in *B. subtilis* protoplasts was obtained, suggesting that the fully displaced nontemplate DNA strand is not an active template for replication *in vivo*. In support of the *in vivo* studies, two lines of evidence have demonstrated that ϕ 29 replication occurs by initiation from two origins at both ends of the same template DNA molecule, and that the two initiation events are largely asynchronous. Firstly, Type II replicative intermediates appear well before full-length products are formed, a necessary condition for full-length ssDNA to be produced (Gutiérrez *et al.*, 1991). Therefore, appearance of these ssDNA molecules able to form a panhandle is apparently not an obligatory step for DNA replication. Secondly, neither *in vivo* (Inciarte *et al.*, 1980), nor *in vitro* (Gutiérrez *et al.*, 1991), have full-length ϕ 29 ssDNA molecules been detected when the natural ϕ 29 template with TP at both ends was used. These results support a model in which initiation of replication can occur from both DNA ends, while Type II molecules are produced by separation of the two displacement forks when they meet.

As with ϕ 29 DNA replication, a sliding-back transition for PRD1 DNA replication has been proposed based on experiments using single-stranded oligonucleotide templates carrying the sequence corresponding to the first 25 bases of the 3' end of PRD1 DNA (Caldentey *et al.*, 1993). The fourth consecutive C residue from the 3' end of the template directs, by base complementarity, the dGMP to be linked to the phage TP in the initiation reaction. Analysis of the transition products suggests that sliding-back occurs stepwise, the fourth base being the directing position during the entire process.

1.4.7 Structure of DNA Polymerases and Terminal Proteins

Structural comparisons of DNA polymerases from TP-containing genomes with those belonging to other groups indicated that the protein-priming function does not correspond to any additional DNA polymerase structural domain (Blanco *et al.*, 1991).

Tps are linked to the termini of their DNA genomes via a phosphodiester bond between the β -OH group of either a serine, threonine or tyrosine residue, and the terminal 5' dNTP. No pattern has emerged as to whether a particular amino acid residue of the TP is linked to a specific dNTP (A, C, G, or T). Any of the three amino acids appear to bind any of the four dNTPs.

A short stretch of conserved amino acid sequence has been found among Tps of adenovirus, PRD1, and bacteriophages ϕ 29, PZA and Nf (Hsieh *et al.*, 1990). The consensus sequence is Tyr-Ser-Arg-Leu-Arg-Thr, or YSRLRT using the amino acid single letter code (Figure 1.2). All the conserved sequence motifs are similarly located in the central position of the whole molecule, except for the adenovirus pTP, where it is located close to the amino-terminus. Site-directed mutagenesis, and functional analysis showed that one of the two arginine residues (Arg₁₇₄) in the PRD1 conserved sequence is critical for the formation of the initiation complex activity of the PRD1 TP (Hsieh *et al.*, 1990). On the basis of these results, Arg₁₇₄ is proposed, at least in part, to represent the binding site for the phosphate group of dGTP.

Comparison of the amino-terminal region of the DNA polymerases of linear plasmids such as *kalilo*, with those of adenovirus and bacteriophages of the ϕ 29 type, demonstrate that the exonucleolytic domain of the first group is preceded by a relatively large amino-terminal domain (Chan *et al.*, 1991). The proximity and co-transcription of TP and DNA polymerase genes on the chromosomes of linear bacteriophages (Savilahti and Bamford, 1987; Escarmís and Salas, 1982), suggests that the large amino-terminal domains of compact eukaryotic linear plasmids could have arisen by fusion of the two genes into a single translational unit, and might function as Tps. Furthermore, the DHBV *P*-gene has been demonstrated to contain both the priming and polymerase activities (see Section 1.4.8).

The amino-terminal domains of plasmid DNA polymerases, and the Tps of adenovirus and linear bacteriophages, have been examined for conserved sequence motifs (Hermanns and Osiewacz, 1992; Chan *et al.*, 1991). At least one common conserved sequence pattern of four amino acids, Ser-Tyr-Lys-Asn (SYKN), was observed in the amino-terminal cryptic domains of the DNA polymerases of the *kalilo*, S-1 and pC1K1 plasmids (Chan *et al.*, 1991) (Figure 1.3). Similar motifs, but with either replacement of at least one of the four

Adenovirus pTP	86	Y S R L RYT	92 (653)
φ29 TP	156	Y S R L R T	161 (266)
PZA TP	156	Y S R L R T	161 (266)
Nf TP	156	Y A R L R T	161 (266)
PRD1 TP	172	Y S R - R T	176 (259)
Consensus		Y S R L R T	

Figure 1.2. Amino acid similarities among the TPs of the human adenovirus 2, the *B. subtilis* bacteriophages φ29, PZA, and Nf, and the *E. coli* bacteriophage PRD1. The single letter amino acid code is used. Numbers refer to position of amino acid sequence in polypeptide chain/predicted ORF, and numbers in brackets refer to total number of amino acids in polypeptide/predicted ORF. Adapted from Hsieh, J.-C. *et al.* (1990).

φ29 TP	151	SKVRSYSR-LRTL EESMEM---RTDP-QYYEK	177 (266)
Adenovirus pTP	81	FDSRAYSR-LRYTELSQPG---HQTV-NWSVM	107 (653)
PRD1 TP	167	TFNGNYSRRT-YTSFDEAF-N-KFMTYDIIID	195 (259)
pGKL1 POL	202	FVSETYSYVIFYAKSIY-FPQPRCV-NNWGNN	231 (995)
pC1K1 POL	66	RELRFYST-SAYTKTHW---NNKDR-INKWMQ	92 (1,097)
<i>kalilo</i> POL	173	KPTSNPSIKTKYEAHKS---NIK-RNKNINLS	200 (970)
S-1 POL	182	PKMGRRS-KRRYQSYIPVD---KTEMKNKTLF	209 (929)
pAI2 POL	376	LKTIEGTKYANYTFPIKKDIVVKD--INKKIN	405 (1,202)
Consensus		-----S-----Y-----K-----N----- T R Q	

Figure 1.3. Amino acid similarities among TPs and putative DNA polymerases (POL) with large, cryptic, amino-terminal domains. The single letter amino acid code is used. Bars indicate identical residues, and circles represent a conservative difference. Numbers refer to position of amino acid sequence in polypeptide chain/predicted ORF, and numbers in brackets refer to total number of amino acids in polypeptide/predicted ORF. Adapted from Chan, B. S.-S. *et al.* (1991).

amino acids by a functionally related amino acid, or missing the asparagine, could be identified in the amino-terminal domain of the DNA polymerase of the *K. lactis* K1 plasmid, in the pTP of adenoviruses, and in the TPs of the linear bacteriophages. However, there is weakness in this analysis. Firstly, there is no structural or genetic data to support the idea that the SYKN motif is relevant to the function of any known TP. Secondly, there is a possibility that the SYKN motif was observed by chance, rather than as a feature that was evolutionarily conserved, since some of the proteins are very rich in amino acids with hydroxyl side chains. It should be noted that the SYKN and YSRLRT motifs overlap. The serine residue of the SYKN motif is the same serine residue of the YSRLRT motif.

With the exception of the YSRLRT motif discussed above, the primary sequences of the TPs of adenovirus, ϕ 29 and PRD1 do not show any significant sequence similarity (Hsieh *et al.*, 1990; Savilahti and Bamford, 1987). However, some weak amino acid sequence similarity between the TPs of adenovirus and PRD1, in the regions containing the site of linkage to the 5' termini, has been observed (Shiue *et al.*, 1991).

Predicted secondary structures for the regions around the DNA-linking amino acid residues in the TPs of adenovirus, ϕ 29 and PRD1 have also been compared (Shiue *et al.*, 1991). The linking Tyr₁₉₀ residue of PRD1 is found in a β -sheet after an α -helix in a hydrophobic area. Interestingly, Arg₁₇₄, proposed to be the binding site for the phosphate group of dGTP (Hsieh *et al.*, 1990), is close to Tyr₁₉₀. The DNA-linking Ser₂₃₂ residue of the ϕ 29 TP and the DNA-linking Ser₅₈₀ residue of the adenovirus 2 TP both lie in β -turns located after α -helix regions in a hydrophilic area (Hermoso *et al.*, 1985; Smart and Stillman, 1982). The significance of this data, bearing in mind that no structural data exist for any of these TPs, is not understood. The TPs would appear to function as a complex with their respective DNA polymerase. Therefore, it is possible that the secondary and tertiary structures of the free TPs are quite different from those of functional complexes.

The TPs of bacteriophages M2 and ϕ 29 contain an Arg-Gly-Asp (RGD) motif (Kobayashi *et al.*, 1989). Replication of M2 was studied *in vitro* using three purified components: the free TP, known as the primer protein (PP), the M2 DNA polymerase, and template DNA linked to TP (Kobayashi *et al.*, 1991). The PP competed with a synthetic RGD peptide for binding to the template DNA-TP complex. Also, the RGD receptor site was shown to be active in TP, but not in PP, in spite of them having the same primary structure. Upon linking a deoxynucleotide to PP, PP-dNTP bound to both the RGD peptide and PP. These results demonstrate that the RGD motif of PP is responsible for the interaction of the PP-DNA polymerase complex with template DNA, and that this motif within the complex recognises the RGD receptor site of the TP. Presumably, when the first nucleotide is linked in the priming reaction, PP loses its affinity for the DNA polymerase, which can then proceed to elongate the DNA chain, and there is a conformational change in PP, converting

it to TP, resulting in the exposure of the RGD receptor site for the next round of DNA replication. Using site-specific mutations in the nucleotide sequence of PP, in an *in vitro* DNA replication system, the RGD motif of M2 was also shown to be important for binding the TP, and for the initiation of protein-primed DNA replication (Kishi *et al.*, 1994).

1.4.8 Hepadnaviruses

Hepatitis B viruses, also called hepadnaviruses, contain a small (3 kb), partially dsDNA genome which is held in circular conformation by base-pairing between the overlapping 5' ends of the DNA strands (Sattler and Robinson, 1979; Summers *et al.*, 1975). Hepadnaviruses replicate their DNA genomes by synthesis of an unspliced RNA intermediate, called the pre-genome, that is packaged into a viral nucleocapsid and reverse transcribed into a DNA genome (Büscher *et al.*, 1985; Summers and Mason, 1982).

Human hepatitis B virus (HBV), ground squirrel hepatitis virus (GSHV), and duck hepatitis B virus (DHBV) have been shown to contain a protein covalently linked at the 5' end of the complete (-) viral DNA strand (Molnar-Kimber *et al.*, 1983; Weiser *et al.*, 1983; Gerlich and Robinson, 1980), suggesting that hepadnaviral reverse transcription is primed by a protein. The DNA-protein linkage in DHBV is a phosphodiester bond between a tyrosine residue and dGMP (Weber *et al.*, 1994; Zoulim and Seeger, 1994; Molnar-Kimber *et al.*, 1983). The protein was also bound to (-) strand DNA intermediates, as small as 30 bases, isolated from replicating complexes present in infected duck liver, supporting the idea that the protein may function as a primer for synthesis of the minus strand DNA (Molnar-Kimber *et al.*, 1983), and thereby substitute for the tRNA used for DNA synthesis by all known retroviral reverse transcriptases (Bartenschlager and Schaller, 1988; Molnar-Kimber *et al.*, 1983).

The *P*-gene is present in all hepadnaviruses. Its protein product shows regions of amino acid similarity with the DNA polymerase and RNase H domains encoded by retroviruses (Radziwell *et al.*, 1990; Khudyakov and Makhov, 1989; Toh *et al.*, 1983). In addition, detailed mutational analysis of the origin for reverse transcription of minus strand DNA indicated that the signals required for DNA synthesis and for attachment of protein to DNA are genetically inseparable (Seeger and Maragos, 1991; Seeger and Maragos, 1990), suggesting that the DNA polymerase and priming functions are encoded by the same protein. Indeed, biochemical and serological data have made it evident that the DHBV primer protein forms a distinct domain comprising the amino terminal quarter of the 785 amino acid long multifunctional *P*-gene product (Bartenschlager and Schaller, 1988). This

domain is separated from the polymerase and RNase H domains, located towards the carboxy-terminus, by a highly variable spacer sequence, which can be manipulated without loss of polymerase activity by insertions or deletions. Using a cell-free rabbit reticulocyte *in vitro* translation system, supplemented with viral RNA encoding the DHBV *P*-gene, it has been demonstrated that the *P*-gene protein can both prime and polymerise viral DNA in RNA-dependent reactions, and remains covalently linked to nascent DNA (Wang and Seeger, 1992).

1.4.9 RNA Viruses with VPg

Certain groups of animal and plant viruses, such as picornavirus, calicivirus, comovirus, and luteovirus, have been shown to contain a genome-linked viral protein (VPg) attached to the 5' end of the single-stranded RNA genome, or the (-) strand of double-stranded RNA genomes (Vartapetian and Bogdanov, 1987; Daubert and Bruening, 1984; Wimmer, 1982). The best studied example is the dsRNA genome of poliovirus.

VPg was found to be covalently linked to the 5' end of poliovirus RNA (Lee *et al.*, 1977; Flanagan *et al.*, 1977), to nascent strands of poliovirus intermediates, and to poly(U) of (-) strands (Flanagan *et al.*, 1977; Nomoto *et al.*, 1977; Pettersson *et al.*, 1978). A tyrosine residue, located at position 3 from the amino-end of VPg, is covalently linked to dUMP (Kitamura *et al.*, 1981). A mechanism for initiation of poliovirus RNA synthesis has been proposed in which either VPg, a polypeptide precursor to VPg, or a uridylylated derivative of VPg acts as primer for the viral RNA polymerase (Takeda *et al.*, 1986; Morrow *et al.*, 1984; Takegami *et al.*, 1983). Antibodies against VPg immunoprecipitate VPg as well as VPgpU(pU) from infected cells (Crawford and Baltimore, 1983), and in *in vitro* replication reactions, anti-VPg antibody specifically inhibits initiation of viral RNA synthesis (Morrow and Dasgupta, 1983; Baron and Baltimore, 1982). The *in vitro* synthesis of VPgpU and VPgpU(pU) was demonstrated in a membraneous replication complex, isolated from infected HeLa cells (Takeda *et al.*, 1986; Takegami *et al.*, 1983).

The synthesis of VPgpU(pU), as is the case of full-length viral RNA, is strictly dependent on the presence of intact membranes (Takeda *et al.*, 1986). Since VPg is a strongly basic protein, it must be delivered to the hydrophobic site by a lipophilic carrier. A molecular genetic analysis, combined with an *in vitro* biochemical approach, supports a model that employs a viral membrane protein as carrier for VPg in the initiation of (+) strand RNA synthesis (Giachetti and Semler, 1991). This data also suggests that a separate mechanism could be used in the initiation of (-) strand RNA synthesis.

1.4.10 Other Proteins that Form Covalent Linkages to DNA

A number of other systems have been characterised where a protein becomes covalently linked to a DNA molecule. The gpA protein of bacteriophage ϕ X174 introduces a specific nick into the (+) strand of the supercoiled ϕ X174 genome to initiate rolling-circle replication. The G residue at the 3'-OH end of the nick site forms the primer terminus (Eisenberg *et al.*, 1976; Ikeda *et al.*, 1977; Langeveld *et al.*, 1978), and the protein covalently attaches, via a tyrosine residue, to the 5'-phosphate group (Eisenberg *et al.*, 1977; Ikeda *et al.*, 1979). After a round of (+) strand synthesis, gpA, bound to the 5' end of the displaced strand, cleaves the origin sequence that has been regenerated during replication. The second cleavage releases the displaced single strand from the replication complex generating a new 3'-OH primer and, simultaneously, the gpA ligates the 3' and 5' ends of the displaced strand to generate a (+) strand circle.

GpA has dual active sites that participate in the covalent protein-DNA intermediate (van Mansfield *et al.*, 1986). Two tyrosine residues, separated by three amino acids in the sequence Tyr-Val-Ala-Lys-Tyr-Val-Asn-Lys, are linked to the DNA with equal efficiencies, and probably act in alternation during the multiple rounds of cleavage and ligation. The RepC protein of the Gram-positive bacterial plasmid pT181 is presumed to have a similar mechanism of action to that of gpA (Murray *et al.*, 1989).

The reaction mechanism of topoisomerases also involves the formation of a protein-DNA intermediate via a phosphotyrosine bond (Eng *et al.*, 1989; Worland and Wang, 1989; Rau *et al.*, 1987; Shuman and Moss, 1987; Huang, 1986; Maxwell and Gellert, 1984; Kirkegaard and Wang, 1985; Tse *et al.*, 1980). The gpA and RepC proteins are sequence-specific type I topoisomerases by virtue of being able to nick and reseal DNA at the origin of replication.

The DNA genome of autonomous parvoviruses, also known as adeno-associated viruses (AAVs), contain palindromic sequences at their termini that allow the formation of a hairpin loop structure, and thereby provide a base-paired 3'-OH primer (Lusby *et al.*, 1981; Lusby *et al.*, 1980). The 5' termini are replicated by a hairpin-loop transfer mechanism, which involves site-specific cleavage of a replicative intermediate by a viral-encoded enzyme. Covalent attachment of the enzyme to the cleavage site resembles the actions of gpA in ϕ X174 replication (Im and Muzyczka, 1990).

Bacterial conjugation also provides a further example of the formation of a covalent bond between protein and DNA. The transfer of DNA is initiated by nicking at a specific plasmid sequence (Willetts and Wilkins, 1984), and one of the proteins present in these

complexes becomes covalently attached to the 5' end of the nicked DNA (Blair and Helinski, 1975; Guiney and Helinski, 1975).

1.5 THE *KLUYVEROMYCES LACTIS* LINEAR KILLER PLASMIDS

1.5.1 Physical Structure and Localisation

Restriction enzyme analysis and electron microscopic analysis (Wésolowski *et al.*, 1982b; Gunge *et al.*, 1981) of gel-purified plasmids has revealed K1 and K2 to be linear dsDNA molecules of 8.9 kb and 13.4 kb, respectively. Buoyant densities of the plasmid DNA were determined by analytical centrifugation in neutral CsCl gradients (Gunge *et al.*, 1981). Both showed an identical density of 1.687 g cm^{-3} , lower than that of both *K. lactis* host nuclear (1.699 g cm^{-3}) and mitochondrial (1.692 g cm^{-3}) DNA, implying that the plasmids have a high A/T content of about 73%. From densitometric measurement of total DNA subjected to agarose gel electrophoresis, it is estimated that the plasmids are present at between 50 and 100 copies per haploid cell (Gunge *et al.*, 1982).

That the plasmids have proteins covalently attached to their 5' termini was first suggested by work which showed that both K1 and K2 are resistant to digestion by *lambda* exonuclease (a 5' to 3' exonuclease), but not to exonuclease III (a 3' to 5' exonuclease) (Kikuchi *et al.*, 1984). Furthermore, the 5' ends of the plasmids are not substrates for T4 polynucleotide kinase, even after treatment with alkaline phosphatase (Kikuchi *et al.*, 1984). These properties clearly suggested that both K1 and K2 have free 3' ends and blocked 5' ends. The finding that terminal restriction fragments treated with pronase E, proteinase K, or trypsin, exhibit shifts in their electrophoretic mobilities through polyacrylamide gels pointed to the presence of covalently attached protein (Kikuchi *et al.*, 1984). That proteins are attached to the 5' termini of the plasmids was demonstrated directly by radio-iodination of terminal restriction fragments, treating them with S1 nuclease to digest the DNA, and then performing SDS-PAGE to visualise the proteins (Stam *et al.*, 1986). This analysis estimated the molecular weights of these proteins at 28 kDa and 36 kDa for K1 and K2, respectively.

The linear plasmids appear to be located in the yeast cytoplasm. Initial evidence for this came following the transfer of the plasmids to a strain of *S. cerevisiae* that lacked mitochondrial DNA (Gunge *et al.*, 1982). When the parent and the transformant were stained with a fluorescent dye, DAPI (4',6-diamidino-2-phenylindole), the former showed a single large fluorescent body corresponding to the nucleus. No staining was detected in the cytoplasm, whereas the transformant was shown to contain numerous small cytoplasmic bodies in addition to the nucleus. Further evidence for a cytoplasmic location

for the linear plasmids comes from cell fractionation studies (Stam *et al.*, 1986). *K. lactis* spheroplasts were osmotically lysed to leave nuclei and mitochondria largely intact. On removing unlysed cells, cellular organelles and debris by differential centrifugation, at least 95% of linear plasmids were found in the cytoplasmic fraction.

An extranuclear location for K1 and K2 is consistent with the high A/T content of the plasmids. It is thought that the high A/T content of mtDNA is a consequence of a lack of access to uracil excision and repair mechanisms present within the nucleus (Dujon, 1981). Together with the data on replication and expression discussed in Sections 1.5.4 and 1.5.5, the above evidence points very strongly towards a cytoplasmic location for the linear plasmids.

The plasmids show a curious incompatibility with mtDNA in *S. cerevisiae* (Gunge and Yamane, 1984). Spheroplast fusion has been used to introduce the plasmids into *S. cerevisiae*. Many [*rho*⁰] strains of *S. cerevisiae* maintain the plasmids stably, whereas all [*rho*⁻] and [*rho*⁺] strains tested do not. The instability was not eliminated by chloramphenicol, indicating that mitochondrial protein synthesis was probably not responsible for this incompatibility. The incompatibility may occur because the killer plasmids and mtDNA have some common replication or segregation factors. However, the killer plasmids are not incompatible with *K. lactis* mtDNA. The incompatibility may therefore arise from an indirect effect of the heterologous nature of the host.

1.5.2 Genetic Organisation

The complete nucleotide sequence has been determined for both K1 and K2. This has confirmed the buoyant density data of an overall composition of 73% A/T for both plasmids. Sequencing of K1 (Stark, 1988; Sor and Fukuhara, 1985; Hishinuma *et al.*, 1984; Stark *et al.*, 1984) indicated a total length of 8,874 bp and revealed the presence of TIR sequences of 202 bp and four large open reading frames (ORFs), as shown in Figure 1.4. The genome organisation is very compact; only 74 out of 8,470 of unique sequence appear to be non-coding. Indeed, ORF2 and ORF3 overlap in frame on opposite strands for 12 codons. Transcripts corresponding to each ORF have been detected, indicating that each ORF is an expressed gene (Sor and Fukuhara, 1985; Stark *et al.*, 1984).

Sequencing of K2 (Tommasino *et al.*, 1988; Wilson and Meacock, 1988; Hishinuma *et al.*, 1984; Sor *et al.*, 1983) indicated a total length of 13,457 bp, and revealed the presence of TIR sequences of 182 bp, unrelated to those of K1, plus ten ORFs, as shown in Figure 1.4. As with K1, the genome organisation is very compact, with the ORFs occupying over 97% of the plasmid. The amino-terminal coding regions of ORF8 and ORF9 diverge from an

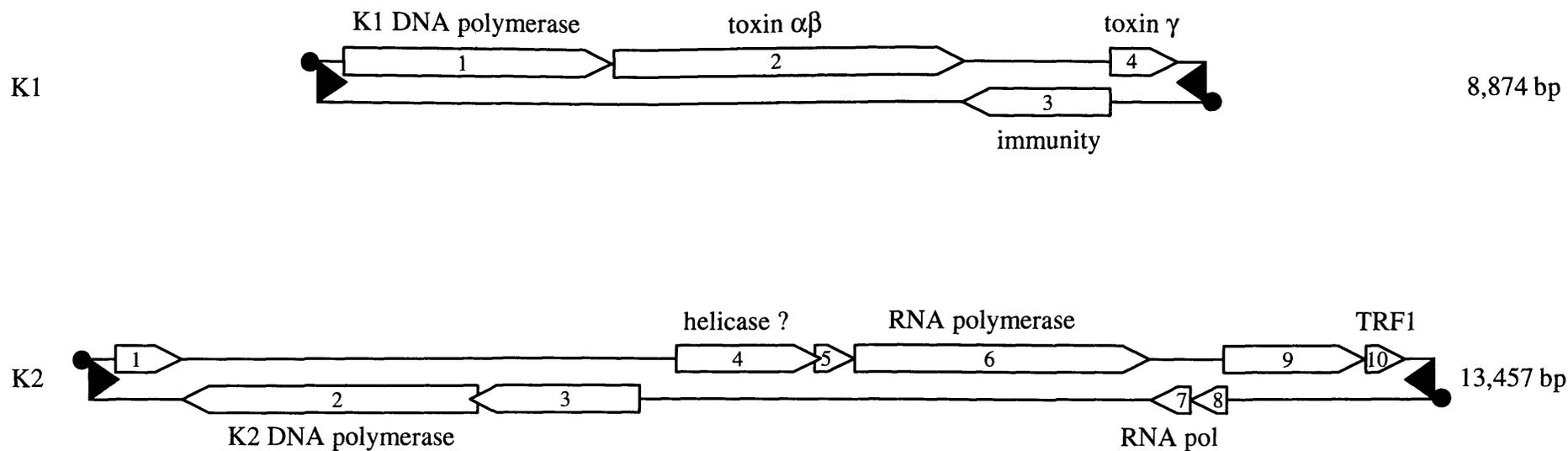


Figure 1.4. Genetic organisation of the *K. lactis* linear plasmids. The ORFs (\square) are labelled according to Sor and Fukuhara (1985) and Tommasino *et al.* (1988), and arrows indicate direction of transcription. Where functions can be assigned, they are indicated. \blacktriangleright represents TIR sequences, while \bullet represents TPs.

intergenic space of 1 bp; ORF1 and ORF2 overlap for 4 bp on opposite strands; and ORFs 2 and 3, and 4 and 5 show partial overlap of 4 to 11 bp in different frames. Specific transcripts have been demonstrated for ORFs 4, 5 and 6 using Northern analysis. However, using a total K2 probe eight distinct RNA species, whose sizes correspond approximately to those of the eight largest ORFs, were detected (Tommasino *et al.*, 1988). C. Fleming and P. Meacock (personal communication) have demonstrated the existence of discrete transcripts corresponding to each of the ten K2 ORFs using primer extension analysis.

1.5.3 The Toxin and Immunity Functions

SDS-PAGE analysis has shown the killer toxin to be comprised of three discrete subunits, termed α , β , and γ , of M_r 99, 30 and 27.5 kDa, respectively (Stark and Boyd, 1986). *K. lactis* strains lacking K1, or lacking both K1 and K2, or containing K1 with a large central deletion (K1-NK2), all fail to produce toxin (Wésolowski *et al.*, 1982a; Niwa *et al.*, 1981). This, together with amino-terminal sequence analysis of purified toxin subunits (Stark and Boyd, 1986), has revealed the toxin to be K1-encoded. K1 ORF2 encodes the α and β subunits while K1 ORF4 encodes the γ subunit. Both K1 ORFs 2 and 4 have the distinct hydrophobic signal sequences typical of secreted polypeptides.

The inhibitory activity of the toxin resides solely in the γ subunit, since intracellular expression of the γ polypeptide in *S. cerevisiae* cells prevents colony formation (Stark and Boyd, 1986). The toxin causes sensitive yeast to arrest proliferation as unbudded cells during the G1 phase of the cell division cycle (White *et al.*, 1989; Sugisaki *et al.*, 1983). The α subunit shows amino acid sequence similarity to both plant and bacterial chitinases, and the toxin is a potent exochitinase when assayed using the model substrate 4-methylumbelliferyl-(GlcNAc)₂, although it shows negligible activity against chitin itself (Butler *et al.*, 1992). The toxin's chitinase activity is required for its biological activity, which is abolished by allosamidin, a specific chitinase inhibitor (Butler *et al.*, 1992). Given its chitinase activity, the α subunit may be involved in binding the toxin to carbohydrate moieties on the yeast cell surface. The β subunit shows similarity of borderline statistical significance to the *E. coli tolQ (fii)* gene product (Stark *et al.*, 1990). The *tolQ* gene product is involved in the entry of colicins into the cell (Sun and Webster, 1987). This, coupled with the fact that the β subunit is very hydrophobic (Stark, 1990), raises the possibility that it may interact with the cell membrane and promote entry of toxin into the cell.

The expression of an immunity to the secreted toxin also depends on the presence of K1 (Gunge *et al.*, 1981). Strains lacking K1, but which contain K2, are not immune to toxin (Niwa *et al.*, 1981). However, strains containing the K1-NK2 deletion retain immunity

despite not being able to produce toxin (Sor and Fukuhara, 1985; Hishinuma *et al.*, 1984). Also, other deletion derivatives of K1, termed F1 and F2, encode only K1 ORF1 and fail to confer either immunity or toxin production (Kichuchi *et al.*, 1985), implying that the immunity determinant is the product of K1 ORF3. The biochemical basis of immunity is unknown and the predicted K1 ORF3 product shows no significant similarity to any currently known protein sequence. However, it appears to be a cytoplasmic protein since it has neither an amino-terminal hydrophobic signal peptide nor any other strongly hydrophobic regions. Several strains of linear plasmid-free *K. lactis* (Gunge *et al.*, 1981) and of *S. cerevisiae* (Tokunaga *et al.*, 1989) have been reported to carry nuclear genes encoding toxin resistance. This nuclear-encoded resistance is distinct from K1-encoded immunity.

1.5.4 The Replication Apparatus

The cytoplasmic location of K1 and K2, where they would not have access to the nuclear-located replication apparatus of the host cell, and their structural similarity to the genomes of adenovirus and bacteriophage ϕ 29, suggested that they would encode their own replication mechanism. This indeed appears to be the case since each plasmid encodes a protein with strong regions of homology to family B DNA polymerases (Tommasino *et al.*, 1988; Bernard *et al.*, 1987; Fukuhara, 1987; Jung *et al.*, 1987b). This type of polymerase is part of the protein-primed DNA replication systems for adenovirus and ϕ 29, tying in neatly with the presence of TPs covalently attached to the killer plasmids.

The predicted products of K1 ORF1 and K2 ORF2 contain three conserved sequences characteristic of this group of DNA polymerase. As is the case with other family B DNA polymerases, these homology blocks are present in the carboxy-terminal region of the two plasmid ORFs (Jung *et al.*, 1987b). The homology between the K1 ORF1 and K2 ORF2 products is particularly high, having 28% of their residues in common, or 46% if conservative changes are permitted.

It appears that the K2-encoded polymerase cannot function in the replication of K1. The requirement of K1 ORF1 for maintenance of the plasmid has been demonstrated by studies of novel hairpin plasmids derived from K1, termed F2 (Kichuchi *et al.*, 1985) and pGK192S (Gunge and Kitada, 1988; Kitada and Gunge, 1988). Both plasmids are derived from the left-hand end of K1, have a single TIR with a blocked 5' terminus, the two DNA strands at the right hand terminus forming a loop of 4 (F2) or 215 (pGK192S) unpaired bases. The plasmids extend to positions in the K1 sequence where internal inverted repeat sequences occur, and are presumed to have formed by endonucleolytic cleavage of K1, followed by exonucleolytic degradation of the strands from the unprotected 5' ends. The

internal inverted repetition sequence could then make a stable hairpin structure with a single-stranded tail at the 3' end. The unpaired tail would be first removed by single-strand specific 3' to 5' exonuclease, and then polymerisation would occur using the paired 3' end as a primer, thus forming a hairpin plasmid. Plasmid F2 encodes the entire K1 ORF1 sequence and is maintained in cells provided K2 is also present (Kichuchi *et al.*, 1985). The K1 ORF1 sequence of pGK192S is partially deleted and is unable to be maintained unless both K1 and K2 are present (Kitada and Gunge, 1988), showing not only that a functional K1 ORF1 product is needed for plasmid maintenance, but that the K2-encoded polymerase has no function in the replication of K1. This is not surprising since K1 and K2 have different TIR sequences (Sor *et al.*, 1983), and TPs of different molecular weights (Stam *et al.*, 1986).

Each hairpin plasmid is also associated with a second linear plasmid which is an inverted dimer of the hairpin form. This is seen as consistent with a model whereby plasmid replication occurs by a protein-priming and strand displacement mechanism (Kichuchi *et al.*, 1985).

The genes encoding the TPs have not been identified. However, the DNA polymerases contain a relatively large cryptic amino-terminal domain, and the K1 DNA polymerase contains a sequence similar to the SYKN motif described in Section 1.4.7. A sequence in the amino-terminal region of the K2 DNA polymerase has also been aligned with TPs containing the YSRLRT conserved sequence, and other DNA polymerases with large, cryptic, amino-terminal domains (Hermanns and Osiewacz, 1992).

Genomes that contain a TP have some sequence repetition at their DNA ends. The 3' terminal nucleotide sequence of K1 is 3' TGTG, and for K2 is 3' TTTT. Extrapolation of the jumping-back and sliding-back mechanisms proposed for adenovirus and ϕ 29 DNA replication (see Section 1.4.6), suggests that these mechanisms could be involved in killer plasmid replication.

The highly basic character of the K2 ORF10 product (22.3% lysine + 2.9% arginine) and the observed sequence homology to the family of histone-like proteins (Tommasino, 1991), suggest that its product may be a DNA binding protein. K2 ORF10 has been expressed in *E. coli* as a fusion protein, and a specific antibody has been raised (Tommasino, 1991). The protein can bind a DNA-sepharose column with good affinity, and immunoprecipitation and Western/Southern blot experiments demonstrated that the K2 ORF10 product interacts directly with K1 and K2. Northern analysis indicated that K2 ORF10 is one of the most highly transcribed genes of the killer plasmids and the K2 ORF10 product is easily detected in total yeast protein by Western blot. Using cytoplasmic extracts of yeast harbouring the killer plasmids in a gel retardation assay with deletion

mutants of terminal DNA fragments of K1 and K2, an activity was identified that recognises the TIRs of both plasmids (McNeel and Tamanoi, 1991). This activity, termed TRF1 (for terminal region recognition factor 1), recognised base pairs 107-183 within the TIR of K1, and base pairs 126-179 within the TIR of K2. By expressing K2 ORFs 1, 5, and 10 in *E. coli*, and testing extracts for binding activity in the gel retardation assay, K2 ORF10 was found to encode TRF1. Consequently, the K2 ORF10 gene product may play a similar role to the host proteins NF1 and NFIII of adenovirus (see Section 1.4.5), and the viral p6 protein of $\phi 29$ (see Section 1.4.3), in the initiation of replication of K1 and K2. Gel retardation experiments with a DNA fragment containing nucleotides 22 to 268 from the left terminus of K1, termed P_{K1}, have also demonstrated specific interactions of protein(s) present in *K. lactis* harbouring plasmid K2 (Chen *et al.*, 1991). In the absence of K2, binding could be detected to a host protein, though to a lesser extent.

Fragments of both K1 and K2 have been isolated which have autonomously replicating sequence (ARS) activity. Circular autonomously replicating plasmids were recovered following the transformation of *S. cerevisiae* and *K. lactis* with circular or linear DNA molecules containing fragments of K1 DNA ligated with a suitable marker (de Louvencourt *et al.*, 1983; Thompson and Oliver, 1986). YRp-like molecules were also recovered when a yeast nuclear marker was ligated to K2 termini with covalently attached TP (Fujimura *et al.*, 1987). Like YRp plasmids, the circular derivatives were unstable, and K1-derived recombinants were not dependent on K2 for their maintenance. Consensus ARS elements are A/T rich and so it is most likely that the occurrence of the sequences in the killer plasmids is fortuitous, rather than having a functional role in their replication.

1.5.5 Expression of Linear Plasmid Genes

The cytoplasmic location of K1 and K2 (where they may not have access to the nuclear transcription apparatus), the extremely tight packing of their genes, the absence of recognisable yeast nuclear promoter elements, and the high A/T content shown by the ORFs, all suggest that these plasmids have a specialised transcription mechanism. K1 ORFs 1, 2 and 4 gave rise to aberrant transcripts when cloned on *K. lactis* nuclear vectors (de Louvencourt *et al.*, 1983), and no β -galactosidase activity was detected when a *lacZ* fusion was used to quantitate the level of expression of cloned K1 ORF2 in strains of *K. lactis* and *S. cerevisiae* which contain K1 and K2 (Wilson and Meacock, 1988). Thus it appears that the yeast nuclear RNA polymerases I, II and III are unable to recognise and correctly transcribe killer plasmid genes (Wilson and Meacock, 1988). This experiment also provided further evidence of an extranuclear location for K1 and K2 as the killer plasmid transcription system could not access the K1 ORF2-*lacZ* fusion carried on the nuclear vector.

Two of the residues (Leu₄₀ and Ile₈₉₇) sequenced in the mature α and β polypeptides of K1 ORF2 do not conform to the rules of yeast mitochondrial translation, but are consistent with the universal genetic code (Stark and Boyd, 1986), and the plasmids are maintained and expressed normally in *S. cerevisiae* [*rho*^o] strains (Gunge *et al.*, 1982; Gunge and Sakaguchi, 1981). This rules out the possibility that they are expressed by the mitochondrial transcriptional and translational machinery.

Such transcriptional and translational activities are almost certainly not encoded by K1 as all four of its genes have been assigned other functions. However, sequencing of K2 has revealed three ORFs which may encode elements of a plasmid transcriptional apparatus. The predicted products of K2 ORFs 6 and 7 show homology to both eukaryotic and prokaryotic DNA-directed RNA polymerases (Wilson and Meacock, 1988; P. Brophy and P. Meacock, personal communication), and K2 ORF6 has been shown to be essential for killer plasmid maintenance or integrity (see Section 1.5.6). That K2 alone appears to encode a plasmid-specific RNA polymerase is sufficient to explain the apparent dependence of K1 on K2.

The polypeptide encoded by K2 ORF4, also shown to be essential for killer plasmid maintenance or integrity (see Section 1.5.6), has sequence similarity to the predicted vaccinia virus polypeptides of 569 and 637 amino acids (Wilson and Meacock, 1988). The smaller product has been identified as a DNA-dependent ATPase, which is believed to unwind the vaccinia duplex during transcription (Broyles and Moss, 1987a; Broyles and Moss, 1987b). It may therefore be that the K2 ORF4 product performs a similar function for the killer plasmids.

Whether or not the plasmid mRNAs are capped at their 5' ends and polyadenylated at their 3' ends remains unknown. Although K1 transcripts are retained on oligo (dT)-cellulose columns (Stark *et al.*, 1984), this may simply reflect the presence of (A)_n tracts within them.

Gene products were hardly detectable (Wilson and Meacock, 1988), or the transcription was aberrant (Romanos and Boyd, 1988), when K1 genes were cloned into a yeast circular vector, and introduced back into yeast cells. The K1 ORF2 promoter did not direct the expression of the *lacZ* gene on a circular plasmid either, even when the native killer plasmids were present in the cell (Wilson and Meacock, 1988). In contrast, it has been reported that K1 ORFs 3 and 4, with their own promoters, could be expressed on yeast circular vectors (Tokunaga *et al.*, 1989; Tokunaga *et al.*, 1987), although the cloned K1 ORF3 could only weakly express the immunity gene (Tokunaga *et al.*, 1987). The kanamycin gene, Km^R, with its own promoter, could not be expressed on the linear K1

plasmid, but that same gene placed behind the K1 ORF1 promoter could be expressed (Tanguy-Rougeau *et al.*, 1990). Similar results were obtained for the yeast chromosomal *LEU2* gene (Kämper *et al.*, 1989b).

A 226 bp fragment, termed P_{K1}, containing most of the left TIR sequence of K1, which is contiguous to the transcribed ORF1, has been examined for promoter activity in the yeast nucleus on circular vectors (Chen *et al.*, 1991). The P_{K1} promoter was strong enough to confer on *K. lactis* cells the *Lac*⁺ and kanamycin resistance phenotype, and was found to be bidirectional. The Km^R gene was not expressed within the linear K1 plasmid, except when the gene was fused with a killer plasmid promoter. It is therefore likely that, although the host coding sequences can be read correctly on the linear plasmid, the host promoters are not recognised by the killer system. This suggests that the killer plasmid transcription system is very specific for its own promoters, in contrast to the yeast host system which appears to be quite permissive for promoter sequences. It is not known whether P_{K1} promoter activity is divergent in the native killer plasmid.

Examination of nucleotide sequences preceding the killer plasmid ORFs has revealed that all K1 and K2 ORFs are preceded by an upstream conserved sequence (UCS), which are considered to be at least a part of the cytoplasmic promoters (Stark *et al.*, 1990; Romanos and Boyd, 1988; Wilson and Meacock, 1988; Sor and Fukuhara, 1985; Stark *et al.*, 1984). The killer plasmid UCS motifs are similar to the UCSs observed on linear plasmid genes from *Saccharomyces kluyveri* and *Pichia acaciae* (Bolen *et al.*, 1994; Hishinuma and Hirai, 1991). The K1 UCS has the sequence ACT^A/TAAATATGA, whereas the K2 UCS has the sequence TA^A/TNTGA. Evidence for promoter activity has been demonstrated by gene disruption analyses (see Section 1.5.6).

1.5.6 Linear Plasmid Recombination and Genetic Manipulation of the Killer Plasmids

Genetic analysis of two non-killer mutants of *K. lactis* led to the conclusion that each of the two strains carried a point mutation in the K1 plasmid, and that recovery of the killer phenotype was restored when the two mutants were crossed was due to recombination between the two plasmids, and not merely to interplasmid complementation (Wésolowski *et al.*, 1982c). Furthermore, an interesting observation was made when pUCW18, consisting of a *TRP1/ARS1* fragment of YRp7 inserted at the *Nde* I site of pUC18, was linearised within the polylinker and transformed into a [K1⁺K2⁺] strain of *K. lactis* (Romanos and Boyd, 1988). High frequencies of transformation were observed, but most of the transformants were unstable, suggesting that the Trp⁺ phenotype was plasmid borne. Circular plasmids were rescued in *E. coli*, and restriction analysis and nucleotide sequencing performed. All of the plasmids analysed were slightly different in the extent of

a small deletion in the pUCW18 DNA, but all contained an inserted, entire copy of the K1 plasmid. It would appear that the K1 DNA had ligated to the incoming linearised plasmid DNA, generating circular molecules which were able to replicate in the nucleus due to fortuitous ARS elements present within the K1 DNA sequence.

It is also possible to transplace and disrupt linear plasmid genes by transformation with linearised DNA. Using a linear fragment of DNA carrying the *LEU2* gene flanked by the promoter and termination regions of K1 ORF2, a *S. cerevisiae leu2* [K1⁺K2⁺] strain was transformed to leucine prototrophy (Kämper *et al.*, 1989b). The transformants carried a new 7.3 kb plasmid, pJKL1, with the same physical structure as the native linear plasmids, but K1 ORF2 of plasmid K1 was replaced with the *S. cerevisiae LEU2* gene. Similarly, the Km^R gene has been introduced into the K1 plasmid *in vivo*, by transforming a killer strain of *K. lactis* with the linearised Km^R gene bordered with short sequences encoding the toxin and the K1 ORF1 promoter (Tanguy-Rougeau, 1990). The linear recombinants conferred on the host a high level of resistance to the drug, and were extremely stable under non-selective conditions.

Whether these recombination events occur in the cytoplasm or in the nucleus, or whether they reflect a plasmid-encoded mechanism is unknown. Cytoplasmic recombination could be catalysed by proteins normally destined for transport into the nucleus or mitochondrion. However, if recombination is not occurring in the cytoplasm, then the linear plasmids must also be present within the nucleus. Evidence that the linear plasmids can enter the nucleus emerged from work in which attempts were made to recombine the *S. cerevisiae LEU2* gene into K1 (Kämper *et al.*, 1989a). After integration of the nuclear *LEU2* gene by *in vivo* recombination in a *S. cerevisiae leu2* [K1⁺K2⁺] strain, the linear hybrid molecules obtained had no proteins attached to their 5' ends. However, telomere-specific sequences were added to the ends of K1. Since it is hard to envisage telomeres being located in the cytoplasm, it must be considered highly likely that the linear plasmids can enter the nucleus. However, this does not demonstrate whether integration of the *LEU2* gene into K1 occurs in the cytoplasm or the nucleus.

Using this *in vivo* homologous recombination technology, a *LEU2* gene cartridge, flanked by K2 ORF1 segments, was used to replace the central region of K2 ORF1 in a *K. lactis* strain (Schaffrath *et al.*, 1992). Cultivation under selective conditions on minimal media resulted in the loss of the native K2 plasmid. Only the recombinant plasmid, pRKL2, and the native K1 plasmid remained. This demonstrated that K2 ORF1 is dispensable for the replication and maintenance of the linear plasmids.

It has also been shown that, concomitant with a selectable marker gene, foreign DNA can be integrated into a linear plasmid via homologous recombination. In addition to the *LEU2*

selection marker, a phosphotransferase-encoding bacterial antibiotic resistance gene was integrated into the K1 plasmid (Meinhardt *et al.*, 1994). This work also demonstrated that in phase fusions to the corresponding ORF are not generally required for expression of heterologous genes in linear plasmids. However, efficiency of expression is dramatically enhanced when the 5' noncoding sequences in front of the heterologous genes are the same as those found on the native killer plasmids (Schrunder and Meinhardt, 1995).

Using disruption vectors, employing the *K. lactis* *TRP1* gene fused to the UCS element of K1 ORF2 (K1 UCS2), K2 ORF2 and ORF6 deletion plasmids were constructed (Schaffrath *et al.*, 1995). Neither of the recombinant plasmids were fully functional and able to displace parental K2, indicating that both genes are essential for plasmid maintenance or integrity. Similarly, K2 ORF4 was disrupted using the *E. coli* Tn903-derived *APT1* gene as selectable marker fused to K1 UCS2 (Soond, 1994), and K2 ORF5 was disrupted using the *S. cerevisiae* *LEU2* gene fused to K2 UCS5 (Schaffrath and Meacock, 1995). The recombinant plasmids were unable to displace native K2 under selective conditions, indicating that K2 ORFs 4 and 5 may also encode functions essential for plasmid stability.

By transplacing K2 ORF5 from K2 onto K1, via *in vivo* homologous recombination, it was shown that this gene was functionally interchangeable between the plasmids (Schaffrath and Meacock, 1996). Once transferred onto K1, the K2 ORF5 deletion was fully complemented *in trans*, giving rise to yeast strains containing only the two recombinant plasmids. Using this gene-shuffle system to transplace an epitope-tagged K2 ORF5, the protein product (Orf5p) was easily detected in comparison to TRF1 (Schaffrath and Meacock, 1996), indicating that Orf5p is one of the most abundant K2 products, and implying a structural rather than a regulatory function.

In vivo homologous recombination has also allowed analysis of the UCSs. Using the bacterial glucose dehydrogenase gene as a reporter, expression driven by seven killer plasmid promoters was determined (Schickel *et al.*, 1996). The highest activity was displayed by K2 UCS6, whereas the lowest level was obtained with K2 UCS2. As demonstrated by deletion of K2 UCSs 5 and 10, deletion of the UCSs led to an almost complete loss of expression, but sequences located upstream of the UCSs did not influence expression.

The K2/K1 gene-shuffle system has also been used to investigate killer plasmid promoter function (Schaffrath *et al.*, 1996). Various K2 ORF5 deletion constructs were transplaced onto K1, but only those containing a complete UCS were able to complement the K2 ORF5 deletion *in trans*. This strongly suggested that K2 UCS5 is an essential cis-acting element involved in ORF5 gene function.

Northern blot analysis and phosphor image technology was used for qualitative and quantitative analyses of the *S. cerevisiae* *LEU2* gene fused to UCS5 (Schaffrath *et al.*, 1996), and revealed a plasmid-dependent *LEU2* transcript distinct in size and regulation from its nuclear counterpart. When compared to nuclear mediated transcription, taking into account relative gene copy numbers, the UCS5-driven *LEU2* mRNA levels were found to be reduced five to eightfold per gene copy, suggesting that the K1 and K2 plasmids have evolved a low-level expression state that will not jeopardise the viability of the *K. lactis* host cell. These, and the above data reinforce the idea of the existence of a unique, and highly conserved, expression system for the killer plasmids.

In vivo homologous recombination has also been used to develop the killer plasmids as cytoplasmic expression vectors (Schrunder *et al.*, 1996). Though efficiency of expression was low when compared to bacterial systems, cytoplasmic expression of the *Streptomyces rubiginosus* xylose isomerase and *Streptococcus pyogenes* UDP glucose dehydrogenase proteins, under control of K2 UCS5, was clearly demonstrated.

Difficulties arise in studying killer plasmid gene function as their structure cannot be readily modified by *in vitro* manipulation because of protein-linked termini. Furthermore, the plasmids appear to encode their own transcription mechanism, and their genes are not correctly expressed by the host transcription system when cloned in conventional circular vectors (Wilson and Meacock, 1988; Stam *et al.*, 1986; de Louvencourt, 1983). However, *in vivo* homologous recombination has provided a powerful tool to allow for the disruption and specific modification of their genes.

1.6 AIMS OF STUDY

The data presented herein formed part of a study investigating the novel cytoplasmic DNA replication system of the *K. lactis* linear killer plasmids. Other than deductions made from the DNA sequence, very little is known about the size, function, abundance and location of the protein components of the killer plasmid replication system. The overall aims of the study were to identify plasmid-encoded components involved in the replication of K1 and K2, and to develop an *in vitro* system for the investigation of killer plasmid replication.

This particular investigation concentrated on the isolation of the killer plasmids with intact TPs for use in biochemical, genetic and molecular analysis. The other major part of the study, carried out in parallel to this work, was the identification of the K1 and K2 putative DNA polymerases using antibody techniques, and their overexpression for use in an *in vitro* replication system.

Stam *et al.* (1986), had described a method for the small scale purification of killer plasmids with intact TPs. This method was used as a starting point, although for the experiments anticipated in this study, a large scale, and reliable method was required for their purification. For example, when this study was initiated, the genes encoding the TPs had not been identified. All four genes on K1 had been assigned other functions, and the only way that K1 could encode its own TP was if this function was also encoded by K1 ORF1 and the TP proteolytically cleaved from the DNA polymerase at some point in the replication cycle. Also, the amino-terminal regions of the K1 and K2 DNA polymerases appeared to contain sequences similar to motifs found in TPs and DNA polymerases with large, cryptic, amino-terminal domains (see Section 1.4.7). Hence, it appeared likely that the K1 and K2 TPs were located within the cryptic amino-terminal domains of their DNA polymerases. It was therefore necessary to identify the genes encoding the TPs, and the chosen approach was by direct amino acid sequencing after a reliable isolation procedure for the killer plasmids had been developed.

CHAPTER 2

MATERIALS AND METHODS

2.1 INTRODUCTION

Standard and adapted methods used in this work are described below. Details of some methods are given in 'Chapters 3' to '5'. Standard methods are described briefly, whereas adapted methods, or methods important to this work, are described in more detail. Where methods are not described, details can be found in standard laboratory manuals. The commercial suppliers of reagents and equipment are not provided where the product is commonly available, and where individual sources did not have an effect on the results.

2.2 BUFFERS AND MEDIA

Chromatography buffers were sterilised by filtration using a 'Stainless Screen Glass Filter Holder' (Millipore) and a 0.22 μm pore size 'Durapore Membrane Filter' (Millipore). Depending upon their composition, where necessary, other buffers, reagents and media were sterilised by autoclaving at 15 p.s.i. for 15 min, by autoclaving at 10 p.s.i. for 15 min, or by filtration using a 0.22 μm pore size filter.

Gel loading buffer

0.25% bromophenol blue,
0.25% xylene cyanol,
30% glycerol

For 0.8% agarose/0.2% SDS gel electrophoresis, and PAGE in the presence of SDS, the buffer also contained 1% w/v SDS. 0.2 volumes of the buffer was added to the sample prior to loading.

5 \times Klenow reaction buffer

500 mM Tris-HCl (pH 7.2),
100 mM MgSO_4 ,
1 mM DTT,
500 $\mu\text{g ml}^{-1}$ bovine serum albumin (BSA Pentax Fraction V)

NME buffers

50 mM MOPS,
x M NaCl,
1 mM EDTA,
pH 7.5

0.1 M NME contained 100 mM NaCl, 0.3 M NME contained 400 mM NaCl, 0.4 M NME contained 400 mM NaCl, and 1 M NME contained 1 M NaCl.

S buffer

1.2 M sorbitol,
25 mM KH_2PO_4 ,
pH 7.5

SED buffer

1.2 M sorbitol,
50 mM DTT,
20 mM EDTA

10 × TAE buffer

108 g L^{-1} Tris-HCl,
11.42 ml L^{-1} glacial acetic acid,
4.7 g L^{-1} EDTA,
pH 8

10 × TBE buffer

108 g L^{-1} Tris-HCl,
55 g L^{-1} boric acid,
9.3 g L^{-1} EDTA,
pH 8.3

For the visualisation of killer plasmids on agarose gels, and killer plasmid TRFs by PAGE, SDS was added to a final concentration of 0.2% w/v to 1 × TBE buffer.

TE.1 buffer

50 mM Tris-HCl,
0.1 mM EDTA,
pH 7.5

TM buffer

10 mM Tris-HCl,
10 mM MgCl₂,
pH 8

YPD

10 g L⁻¹ yeast extract,
20 g L⁻¹ peptone,
2% w/v glucose

YPD was used as liquid broth, or as solid medium when supplemented with agar to a final concentration of 2% w/v. Both broth and agar were prepared lacking the carbon source. D-glucose was prepared as a 40% w/v solution, and then the appropriate amount added to the medium prior to use.

SD

6.7 g L⁻¹ yeast nitrogen base,
2% w/v glucose

SD was used as solid medium supplemented with agar to a final concentration of 2% w/v. D-glucose was prepared as a 40% w/v solution, and then the appropriate amount added to the medium prior to use.

2.3 YEAST METHODS

2.3.1 *Kluyveromyces lactis* Strains

IFO1267: *Mata*, prototroph, [K1⁺K2⁺]

SD11: *Mata*, *trp1*, *lac4*, [K1⁺K2⁺]

ABK802: *Mata*, prototroph, [K1⁻K2⁺]

K1-KAN13: *Mata* α , *argA*, *lysA*, *uraA*, [K1⁻, rlpSS8⁺, K2⁺], [pKD1⁺]

2.3.2 Cell Growth and Spheroplast Formation

In developing an isolation procedure for the *K. lactis* linear killer plasmids, it was found that the spheroplasting step was critical. Good lysis was never obtained from poorly spheroplasted cells. Thus, particular attention was devoted to this step in the purification of K1 and K2.

Cell Growth and Harvest

500 ml YPD cultures were grown in 2 L 'Erlenmeyer' flasks in an orbital shaker rotating at 275 rpm, at 30°C. At a density of 2×10^8 cells ml⁻¹, the cells were harvested by centrifugation at 4 K, 20°C, 10 min, in a Sorvall GS3 rotor. The pellet was resuspended in 300 ml Q-water per litre of culture, re-centrifuged and the wet weight of cells determined.

Pre-treatment of Cells

For the pre-treatment of 1 g of wet weight cells:

The cell pellet was resuspended vigorously, rapidly and completely as possible in 3.5 ml SED buffer, and incubated for 1 hr at 30°C with gentle shaking in a water bath. Cells were pelleted by centrifugation at 4 K, 20°C, 5 min, in a Sorvall GS3 rotor, washed with 20 ml S buffer and re-centrifuged.

Spheroplast Formation

For the spheroplasting of 1 g of wet weight cells:

The cells were resuspended vigorously, rapidly and completely in 4 ml S buffer, and CaCl₂ added to a final concentration of 0.4 mM. Yeast Lytic Enzyme (ICN) (40 units), dissolved in a small amount of S buffer, was added per ml of cell suspension, and the tube which contained the enzyme rinsed out with 1 ml of cell suspension. Spheroplasting was performed at 30°C with shaking vigorous enough to keep the cells suspended but gentle enough to avoid turbulence in the flask. After 20 min, two Pasteur pipettes were dipped into the cell suspension and a small volume drawn up into each. One was dispensed into a disposable glass tube containing 10 drops of water, and the other into a tube containing 10 drops of S buffer. If the spheroplast formation was working properly, the suspension in the tube with water would become less turbid. The degree of spheroplasting was estimated by

light microscopy. If the reaction was going too slowly, 25-50% more enzyme was added and the incubation continued. The aim was to spheroplast the cells within 1 hr. Once complete (less than 5% or, preferably, no cells remaining in the water field in the microscope), the spheroplasts were harvested by centrifugation at 2 K, 20°C, 5-10 min, in a GS3 rotor and washed twice with S buffer, using half the spheroplast volume. Spheroplasts being sensitive to mechanical stress were gently resuspended using a lab-spoon spatula. Less than complete resuspension, that is some clumps remaining, was treated as sufficient at this stage. If not used immediately, spheroplasts were stored at -20°C.

2.3.3 Preparation of Electrocompetent Cells

A 500 ml YPD culture of *K. lactis* strain SD11 was grown in a 2 L 'Erlenmeyer' flask in an orbital shaker rotating at 275 rpm, at 30°C. At an A_{600} of 1.3-1.5 (corresponding to a density of approximately 1×10^8 cells ml^{-1}), the cells were harvested by centrifugation at 5 K, 4°C, 5 min, in a Sorvall GS3 rotor. The pellet was washed and re-centrifuged twice, first in a total of 500 ml ice-cold sterile Q-water, and then in a total of 250 ml ice-cold sterile Q-water. The pellet was then resuspended in 20 ml ice-cold 1 M sorbitol, and centrifuged at 5 K, 4°C, 5 min, in a Sorvall SS34 rotor. The pellet was finally resuspended in 0.5 ml ice-cold 1 M sorbitol and stored on ice.

2.4 NUCLEIC ACID METHODS

2.4.1 Agarose Gel Electrophoresis

Agarose gels were prepared from HGT agarose and $1 \times$ TAE buffer, and were electrophoresed at a constant voltage of 20-80 V in $1 \times$ TAE buffer containing $0.5 \mu\text{g ml}^{-1}$ EtBr. Prior to loading, samples were mixed with 0.2 volumes of agarose gel loading buffer. Electrophoresis was normally continued until the bromophenol blue marker dye had reached the end of the gel. DNA bands were visualised by illumination with shortwave UV light. Results were recorded by photography through an orange filter.

0.9% Agarose/In Situ Lysis Gel Electrophoresis

For the visualisation of killer plasmids the gel was cast with two parallel sets of wells 0.5 cm apart, and 8% w/v SDS, in agarose gel loading buffer, was added to the trailing wells. The gel was run, and the DNA bands visualised, as previously described.

0.8% Agarose/0.2% SDS Gel Electrophoresis

For the visualisation of killer plasmids the gel was prepared and run in $1 \times$ TBE buffer containing 0.2% w/v SDS. Prior to loading, samples were mixed with 0.2 volumes of agarose gel loading buffer containing 1% w/v (final concentration = 0.2%) SDS. Otherwise, electrophoresis was as previously described. After electrophoresis, the gel was soaked in Q-water for 45 min to remove SDS, stained with $0.5 \mu\text{g ml}^{-1}$ EtBr in $1 \times$ TBE buffer for 45 min, and the DNA bands were then visualised as normal.

Autoradiography

Gels were soaked in Q-water for 45 min, placed on 'Gelbond' (Pharmacia), and dried using a hair drier. Direct autoradiography was performed at room temperature using 'Hyperfilm-MP' (Amersham) autoradiography film.

2.4.2 Polyacrylamide Gel Electrophoresis (PAGE)

For the preparation of a 40×20 cm polyacrylamide gel, 4 ml of $10 \times$ TBE buffer was mixed with 40% (19:1) acrylamide/bisacrylamide (National Diagnostics) to the desired concentration, $320 \mu\text{l}$ 10% w/v ammonium persulphate, and made up to a final volume of 40 ml with Q-water. The solution was then stirred and $30 \mu\text{l}$ TEMED (*N, N, N', N'*-tetramethylethylene diamine) added. The gel was poured between two glass plates separated by 0.4 mm thick spacers, and the top edge formed using a 1 cm or 0.5 cm slot width comb. When polymerised, the gel was assembled vertically, and run in $1 \times$ TBE buffer at a constant voltage of between 100-200 V. Prior to loading, samples were mixed with 0.2 volumes of gel loading buffer. DNA bands were visualised by direct autoradiography at -70°C using 'Hyperfilm-MP' (Amersham) autoradiography film, or 'NIF RX' (Fuji) X-ray film.

Polyacrylamide Gel Electrophoresis in the Presence of 0.2% SDS

For the visualisation of killer plasmid TRFs, 0.2% w/v SDS was included in the gel mix, and the gel was run in $1 \times$ TBE buffer containing 0.2% w/v SDS. Prior to loading, samples were mixed with 0.2 volumes gel loading buffer containing 0.2% w/v SDS. Otherwise, electrophoresis and visualisation of DNA bands were as previously described.

2.4.3 Digestion with Restriction Endonucleases

Digestions of DNA with restriction endonucleases were performed following manufacturer's instructions, and in the provided or recommended buffers, except for the *Xho* I digestion described below. Killer plasmids were either dialysed into, or ethanol precipitated and resuspended in, the appropriate reaction buffer. The results obtained in experiments with K1 and K2 TRFs (see 'Chapter 5') were the same irrespective of the method used. Restriction endonuclease *Mae* II was obtained from Boehringer Mannheim.

Digestion of Killer Plasmids with Xho I

Approximately 1.5 μg in total of purified K1 + K2, or 0.9 μg K2, was incubated with 5 units of *Xho* I for 3 hr at 37°C. The reaction mixture contained 25 mM MOPS, 50mM NaCl, 10mM MgCl₂, pH 7.5 in a volume of 40 μl . Digestion in this buffer gave the same results for the K2 *Xho* I TRF electrophoretic shifts (see 'Chapter 5') as those produced when the supplied manufacturer's buffer was used.

2.4.4 ³²P 3' End Labelling and Analysis of Killer Plasmid TRFs

The restriction endonuclease-digested sample (1 μg *Msp* I-digested pBR322 DNA; 1.5 μg in total K1 + K2, or 0.9 μg K2; see Section 2.4.3) was ethanol precipitated and resuspended in 27 μl Q-water. ³²P 3' end labelling was performed by adding 8 μl 5 \times Klenow reaction buffer, 1 μl 4 mM G, A, T, 2 μl (2 μCi) [α -³²P]dCTP, and 2 μl (2 units) Klenow fragment of *E. coli* DNA polymerase I, and incubated for 30 min at room temperature. The sample was then ethanol precipitated at room temperature. Killer plasmids were resuspended in 20 μl 0.1 M NME buffer. pBR322 DNA was resuspended in 50 μl TE.1, and 1-5 μl used per lane as marker DNA for PAGE. If not used immediately, samples were stored at -20°C.

Proteinase K Digestion

To 10 μl of the ³²P-labelled killer plasmid TRF sample, 2 μl Q-water, 1.5 μl 2% w/v SDS, and 1.5 μl 10 mg ml⁻¹ proteinase K was added. A control sample contained 10 μl of the labelled sample, 3.5 μl Q-water, and 1.5 μl 2% w/v SDS. Both samples were incubated for 3 hr at 37°C. 5 μl of each sample was used per lane for PAGE.

Treatment with β -mercaptoethanol

To 5 μ l of the 32 P-labelled killer plasmid TRF sample, 3 μ l Q-water, 1 μ l 10% w/v SDS, and 1 μ l 1 M β -mercaptoethanol was added. A control sample contained 5 μ l of the labelled sample, 4 μ l Q-water, and 1 μ l 10% w/v SDS. To 7.5 μ l of the proteinase K-treated 32 P-labelled killer plasmid TRF sample (see above), 0.5 μ l Q-water, 1 μ l 10% w/v SDS, and 1 μ l 1 M β -mercaptoethanol was added. A control sample contained 7.5 μ l of the proteinase K-treated labelled sample, 1.5 μ l Q-water, and 1 μ l 10% w/v SDS. All samples were incubated for 1 hr at 65°C. 5 μ l of each sample was used per lane for PAGE.

Treatment with Piperidine

To 10 μ l of the 32 P-labelled killer plasmid TRF sample, 10 μ l 1 M or 5 M piperidine was added, and incubated for 3 hr at 37°C. 10 μ l Q-water alone was added to a control sample. Both samples were then precipitated with ethanol, and resuspended in 10 μ l 0.1 M NME. 5 μ l of each sample was used per lane for PAGE.

2.4.5 Digestion of the Killer Plasmids with DNase I

1.3 μ g in total of purified K1 + K2 was incubated with 5 units of DNase I (Pharmacia) for 1 hr at 37°C. The reaction mixture contained 30 mM Tris-HCl (pH 7.5), and 6 mM MgCl₂. Control samples, incubated under the same conditions, either did not contain DNase I or the killer plasmids. Samples were analysed by 10% or 15% SDS-PAGE, and stained with silver.

2.4.6 Digestion of the Killer Plasmids with Proteinase K

Approximately 2 μ g in total of purified K1 + K2 in 0.1 M NME containing 0.2% w/v SDS was incubated with 1 mg ml⁻¹ proteinase K for 3 hr at 37°C. A control sample was incubated under the same conditions without the addition of proteinase K. Both samples were then analysed by 0.8% agarose gel electrophoresis.

2.4.7 Digestion of the Killer Plasmids with Exonucleases

Approximately 5 μ g in total of purified K1 + K2 was incubated with exonuclease III and λ exonuclease. The reaction mixture for the exonuclease III digest contained 50 mM Tris-

HCl (pH 7.5), 6 mM MgCl₂, 1 mM DTT, and 20 units exonuclease III, in a volume of 50 µl. The reaction mixture for the λ exonuclease digest contained 50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 6 mM MgCl₂, 1mM DTT, 10 units λ exonuclease, and approximately 5 µg pMJR2120 control DNA (obtained from S. Soond, Department of Genetics, University of Leicester) digested with *Eco* RI, in a volume of 50 µl. After incubation at 37°C, 8 µl of the reaction mixtures were withdrawn into 2 µl gel loading buffer containing 2% w/v SDS at 0, 2, 5, 10 and 20 min, chilled on ice, and analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

2.4.8 Isopycnic Centrifugation of the Killer Plasmids

DE52-purified killer plasmids from a 2 L *K. lactis* strain IFO1267 culture (see 'Appendix I'), were concentrated from 48 ml down to approximately 10 ml using the PEG method described in Section 4.3.1 and 'Appendix I'. The killer plasmids were then mixed with 18.64 g CsCl (starting density = 1.41 g cm⁻³), and made up to a final volume of 34 ml with Q-water. For CsCl gradients containing guanidine hydrochloride, the concentrated linear plasmids were mixed with 18.64 g of CsCl (starting density = 1.41 g cm⁻³) and 17 ml 8 M guanidine hydrochloride (final concentration = 4 M), and made up to 34 ml with Q-water. Gradients were centrifuged at 40 K, 65 hr, 20°C, in a Sorvall TV850 rotor. After centrifugation, the gradients were fractionated by piercing and unloading from the bottom of the centrifuge tube using a home made gradient unloader (School of Biology Workshop, University of Leicester), and a 'Miniplus 3' (Gilson) peristaltic pump.

2.4.9 Sucrose Density Centrifugation of the Killer Plasmids

Gradients were prepared from stock solutions of 10% and 40% w/v sucrose in 1 M NME using a home made gradient maker (School of Biology Workshop, University of Leicester). DE52-purified killer plasmids from a 2 L *K. lactis* strain IFO1267 culture (see 'Appendix I'), were concentrated from 48 ml down to approximately 5 ml using the PEG method described in Section 4.3.1 and 'Appendix I'. The concentrated sample was then loaded onto two 34 ml gradients and centrifuged at 26 K, 24 hr, 10°C, in a Sorvall AH627 rotor. After centrifugation, the gradients were fractionated by piercing and unloading from the bottom of the centrifuge tube using a home made gradient unloader (School of Biology Workshop, University of Leicester), and a 'Miniplus 3' peristaltic pump.

2.5 PROTEIN METHODS

2.5.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The following solutions were used for SDS-PAGE. The quantities described are for a 15% gel using the 'Mini-Protean II Dual Slab Cell' (Bio-Rad) system. For 10% gels, 3.4 ml of 30% (30:1) acrylamide/bisacrylamide (National Diagnostics) and 1.5 ml Q-water were used in the separating buffer. The volume of buffers were increased accordingly for larger gels. Silver staining of gels was performed using either the Amersham or National Diagnostics silver staining kits following manufacturer's instructions.

Separating gel

5 ml 2 × separating buffer, 5 ml 30% (30:1) acrylamide/bisacrylamide, 250 µl 10% w/v ammonium persulphate, 5 µl TEMED

Stacking gel

2.4 ml 2 × stacking buffer, 1.2 ml 30% (30:1) acrylamide/bisacrylamide, 1.3 ml Q-water, 50 µl 10% w/v ammonium sulphate, 2.5 µl TEMED

2 × separating buffer

0.75 M Tris-HCl, 0.4% w/v SDS, pH 8.8

2 × stacking buffer

0.25 M Tris-HCl, 0.4% w/v SDS, pH 6.8

Running buffer

14.4 g L⁻¹ glycine, 3 g L⁻¹ Tris, 1 g L⁻¹ SDS

2 × sample buffer

0.4% w/v bromophenol blue, 0.2 M β-mercaptoethanol, 2% w/v SDS, 100 mM Tris-HCl, pH 8.5

Coomassie blue stain

2.5 g Coomassie Brilliant Blue R-250 (CBB-R250) (Sigma), 250 ml ethanol, 50 ml glacial acetic acid, 200 ml Q-water

Destain

10% v/v glacial acetic acid, 30% v/v ethanol

Samples were boiled in sample buffer for 3 min prior to loading. Mini-gels were run at a constant voltage of 150 V. Larger gels were run at a constant voltage of 20-200 V.

Molecular Weight Standards

MW-SDS-70L and MW-SDS-200 kits were obtained from Sigma, and used according to manufacturer's instructions. For silver stained gels, a dilution of 1 in 100 was used.

MW-SDS-70L Kit

<u>Protein</u>	<u>kDa</u>
Albumin, bovine plasma	66
Albumin, egg	45
Glyceraldehyde-3-phosphate dehydrogenase	36
Carbonic anhydrase	29
Trypsinogen	24
Trypsin inhibitor	20.1
α -lactalbumin	14.2

MW-SDS-200 Kit

<u>Protein</u>	<u>kDa</u>
Myosin	205
β -galactosidase	116
Phosphorylase B	97.4
Albumin, bovine plasma	66
Albumin, egg	45
Carbonic anhydrase	29

Autoradiography

Gels were fixed and stained with CBB-R250 as normal. Direct autoradiography of dried gels was performed at room temperature using 'Hyperfilm-MP' (Amersham) autoradiography film. Positions of molecular weight standards, the top edge of the stacking and separating gels, and the bottom of the gel, were marked using radioactive ink.

2.5.2 Iodination of the Killer Plasmid TPs

To 7.5 µg in total of purified K1 + K2, or 4.5 µg purified K2, in 100 µl 0.1 M NME, an IODO-BEAD™ (Pierce) was added. 5 µl (0.5 mCi) ¹²⁵I was then added, mixed, and incubated for 30 min at room temperature. After incubation, 400 µl 0.4 M NME was added to each reaction, and these loaded onto a 'PD-10' Sephadex® G-25 M (Pharmacia) column previously equilibrated with 25 ml 0.4 M NME. 7.5 ml 0.4 M NME was then applied to the column, and 0.5 ml fractions collected. The killer plasmids eluted in fractions 6 to 8 (as determined by counting and analysis of an unlabelled sample). These fractions were pooled, precipitated with ethanol, and both samples resuspended in 500 µl TE.1.

2.5.3 Analysis of the Iodinated Killer Plasmid TPs

Treatment with Proteinase K

To 25 µl of the iodinated killer plasmid samples, 3 µl 10 mg ml⁻¹ proteinase K, and 2 µl 3% w/v SDS were added. A control sample contained 25 µl of the iodinated killer plasmids, 3 µl Q-water, and 2 µl 3% w/v SDS. Both samples were incubated for 3 hr at 37°C, and then analysed by 0.8% agarose/0.2% SDS gel electrophoresis and autoradiography.

Treatment with DNase I

10 µl of the labelled killer plasmid samples were incubated with 20 units of DNase I for 1 hr at 37°C. The reaction mixtures contained 30 mM Tris-HCl (pH 7.5), and 6 mM MgCl₂ in a volume of 20 µl. The samples were then analysed by 15% SDS-PAGE and autoradiography.

Treatment with DNase I and Exonuclease III

10 µl of the labelled killer plasmid samples were incubated with 20 units of DNase I and 20 units of exonuclease III (Gibco BRL) for 1 hr at 37°C. The reaction mixtures contained 50 mM Tris-HCl (pH 8), 5 mM MgCl₂, and 1 mM DTT in a volume of 20 µl. The samples were then analysed by 15% SDS-PAGE and autoradiography.

Treatment with Micrococcal Nuclease

10 μ l of the labelled killer plasmid samples were incubated with 20 units of micrococcal nuclease (Pharmacia) for 1 hr at 37°C. The reaction mixtures contained 20 mM Tris-HCl (pH 8), and 1 mM CaCl₂ in a volume of 20 μ l. The samples were then analysed by 15% SDS-PAGE and autoradiography.

Treatment with Mung Bean Nuclease

10 μ l of the labelled killer plasmid samples were incubated with 30 mM sodium acetate (pH 4.6), 50 mM NaCl, and 1 mM ZnCl₂ in a volume of 20 μ l at 95°C for 5 min, and then plunged into a dry-ice/ethanol bath. After allowing the solution to thaw on ice, 200 units of mung bean nuclease (Pharmacia) was added, and incubated for 1 hr at 37°C. The samples were then analysed by 15% SDS-PAGE and autoradiography.

Treatment with S1 Nuclease

10 μ l of the labelled killer plasmid samples were incubated with 30 mM sodium acetate (pH 4.6), 50 mM NaCl, and 1 mM ZnCl₂ in a volume of 20 μ l at 95°C for 5 min, and then plunged into a dry-ice/ethanol bath. After allowing the solution to thaw on ice, 250 units of S1 nuclease (Pharmacia) was added, and incubated for 1 hr at 37°C. The samples were then analysed by 15% SDS-PAGE and autoradiography.

2.6 CHROMATOGRAPHY METHODS

2.6.1 DE52 Slurry Preparation and Column Packing

Pre-swollen DE52 (Whatman) anion exchanger, which does not require pre-cycling, was used. For a 120 ml column, approximately 55 g of pre-swollen DE52 was required. Slurry preparation and column packing were performed in an area free of draughts, direct sunlight and heaters, and are described here for use with MOPS buffers. For a 15 ml bed volume column a 'C 10' (Pharmacia) column with an internal diameter of 10 mm was used, and for a 120 ml bed volume column a 'XK 50' (Pharmacia) column with an internal diameter of 50 mm was used.

Slurry Preparation

Pre-swollen DE52 was stirred with 6 ml 1 M NME buffer g^{-1} of ion exchanger, and the pH value adjusted to pH 7.5. The slurry was then allowed to settle, and the supernatant decanted. The DE52 was redispersed in 6 ml 1 M NME buffer g^{-1} ion exchanger, and allowed to settle. The supernatant was then decanted so that the final volume was the volume occupied by the ion exchanger after settling plus 20%.

Column Packing

The column was set up vertically, and the slurry poured into the column. From this moment operations were carried out as quickly as possible. The eluent from the column was allowed to run to waste, and when all the slurry had been added, the top column end was inserted. A 'P-1' (Pharmacia) peristaltic pump was then connected, and 1 M NME buffer pumped through the column at a flow rate of approximately $45 \text{ ml hr}^{-1} \text{ cm}^{-2}$ of the internal cross-sectional area of the column, until the bed height was constant. After column packing the ion exchanger was transferred to a 4°C cold room, and equilibrated with approximately 4 bed volumes of 0.1 M NME at the desired flow rate.

2.6.2 Sephacryl S-1000 Column Packing

Sephacryl[®] S-1000 Superfine (Pharmacia) was supplied as an aqueous suspension, and approximately 480 ml was used for a 320 ml column. After allowing the Sephacryl to settle, excess water was decanted, and the Sephacryl resuspended in approximately 150 ml 1 M NME buffer. A 'C26' (Pharmacia) column, with an internal diameter of 26 mm, was mounted vertically with an 'RC 16' (Pharmacia) extension reservoir fitted, in an area free of draughts, direct sunlight and heaters. The walls of the column were wetted with buffer, and then drained leaving approximately 1 cm of buffer. The slurry was then carefully poured into the column/extension reservoir. From this moment operations were carried out as quickly as possible. The extension reservoir was carefully filled with buffer, a 'P-1' peristaltic pump connected, and the gel packed at a flow rate of approximately 2 ml min^{-1} . After two bed volumes had been applied, the gel reservoir was removed, and a flow adaptor inserted just touching the upper gel surface. The column was then transferred to a 4°C cold room, and two bed volumes of buffer pumped through the column at a flow rate of 0.4 ml min^{-1} .

2.6.3 Chromatography of the DE52-Purified Killer Plasmids using Qiagen®

The manufacturer's supplied buffers were used. 48 ml of DE52-purified killer plasmids from a 2 L *K. lactis* strain IFO1267 culture (see 'Appendix I'), were loaded onto a Qiagen-tip 500, previously equilibrated with 10 ml Buffer QBT (750 mM NaCl, 50 mM MOPS, 15% ethanol, 0.15% Triton X-100, pH 7). The Qiagen-tip was then washed with 6 × 10 ml Buffer QC (1 M NaCl, 50 mM MOPS, 15% ethanol, pH 7), and K1 and K2 eluted with 15 ml Buffer QF (1.25 M NaCl, 50 mM Tris-HCl, 15% ethanol, pH 8.5).

2.7 MISCELLANEOUS METHODS

2.7.1 Preparation of Killer Plasmid TPs for Amino-Terminal Sequencing

Eight 4 L *K. lactis* strain IFO1267 cultures were processed as described in 'Appendix I', and the PEG-concentrated killer plasmid preparations stored at -20°C until required. After the final preparation, the purified killer plasmids were pooled (approximately 20 ml), and dialysed against three changes of 1 L 0.1 M NME at 1 hourly intervals, at 4°C, before precipitating with ethanol. The precipitate was centrifuged at 15 K, 4°C, 30 min, in a Sorvall SS-34 rotor, washed with 70% v/v ethanol and re-centrifuged. The killer plasmid pellet was resuspended in 800 µl 20 mM Tris-HCl, 1 mM CaCl₂, pH 8, and stored at -20°C until required. 20 µl of the sample was retained for analysis. 10 µl of the retained sample was used to record the absorbance at 260 nm, and the yield of killer plasmid DNA was determined to be approximately 3.5 mg, representing 0.48 nmol of each TP, or 12.5 and 14.5 µg of the K1 and K2 TPs, respectively. 0.5 µl of the retained sample was used for the electrophoretic shift analysis of K2 *Xho* I TRFs, and the expected bands were observed for the untreated and proteinase K-treated fragments (see Sections 5.2.3 and 5.4). 2 µl of the retained sample was treated with proteinase K, and analysed by 0.8% agarose gel electrophoresis alongside 2 µl of the untreated sample. The proteinase K-treated sample migrated into the gel, but the untreated sample remained in the sample well. After performing these analyses, 200 units (approximately 10 µg) of micrococcal nuclease was added to the killer plasmid suspension, incubated for 3 hr at 37°C, and then placed on ice. 2.5 µl of the digested sample was analysed by 0.8% agarose/0.2% SDS gel electrophoresis alongside 2.5 µl of the retained sample. The plasmids in the retained sample appeared intact, but no plasmids were visible in the digested sample. The digested sample was then concentrated using two Ultrafree-MC Filter Units (Millipore), with a 5,000 NMWL low-binding, PLCC membrane. The units were centrifuged at 2 K, 4°C, in a microcentrifuge. After approximately 3 hr, the volume of each sample was 200 µl. 200 µl 0.1 M NME was added and the samples re-centrifuged. The washing and centrifuging steps were repeated

twice more, taking the same time. The two samples were combined after the final wash, and concentrated further. After 12 hr the volume of the sample was 40 μ l. A control sample containing 10 μ g of micrococcal nuclease in the same buffer took approximately 1 hr for each of the concentrating and washing steps, and less than 3 hr to concentrate to a final volume of approximately 40 μ l. Both samples, and an untreated micrococcal nuclease (200 units) sample, were then given to J. Kyte, Department of Biochemistry, University of Nottingham, for electrophoresis, blotting, and amino-terminal sequencing.

2.7.2 Preparation of RNase A that is Free of DNase

RNase A (Boehringer Mannheim) was dissolved at a concentration of 10 mg ml⁻¹ in 10 mM Tris-HCl (pH 7.5) and 15 mM NaCl, and incubated for 15 min at 100°C. The solution was then allowed to cool slowly to room temperature, dispensed into aliquots, and stored at -20°C.

2.7.3 Preparation and Use of Geneticin

The sulphate salt of Geneticin® (G418) (Gibco BRL) was dissolved in Q-water at a concentration of 100 mg ml⁻¹, sterilised by filtration, dispensed into aliquots, and stored at -20°C. Geneticin was used in YPD agar at a concentration of 125 μ g ml⁻¹.

CHAPTER 3

EVALUATION OF CELL LYSIS AND KILLER PLASMID RECOVERY METHODS

3.1 INTRODUCTION

For the purposes of amino-terminal sequencing of the K1 and K2 TPs, *in vitro* analysis of killer plasmid replication, and other studies, a reliable procedure for the purification of killer plasmids with intact, and for some studies non-denatured, TPs is required. The following two chapters deal with the difficulties in the isolation of killer plasmid DNA with intact TPs, and the development of a new, large scale, purification procedure. *K. lactis* strain IFO1267 (K1⁺K2⁺) was used for these experiments.

3.1.1 Difficulties in the Purification of the *Kluyveromyces lactis* Linear Killer Plasmids

Standard isolation methods for the *K. lactis* killer plasmids yield plasmids with degraded TPs, and most of the killer DNA is lost during a particular step in these preparations. The standard isolation procedures involve total lysis of *K. lactis* spheroplasts with 1% w/v SDS, followed by a high salt precipitation step to remove cellular debris, protein, and high molecular weight DNA (Gunge *et al.*, 1981). This study (Figure 3.1), and others (Stam *et al.*, 1986), have shown that a large proportion of the killer plasmid DNA is lost at this stage in the salt precipitate. The killer plasmids appear to be precipitated due to the presence of their TPs (Wilson, 1988; see Section 4.3.3), and therefore killer plasmids with degraded TPs are preferentially selected. Furthermore, the method is not effective at removing all the higher molecular weight DNA. K1 and K2 are subsequently purified by agarose gel electrophoresis.

Stam *et al.* (1986) have reported successful preparation of killer plasmids with intact TPs using a protocol based on the observation that the killer plasmids are located in the yeast cytoplasm. *K. lactis* spheroplasts were osmotically lysed, leaving nuclei and mitochondria largely intact. Together with unlysed cells, cellular debris, contaminating chromosomal DNA, and mitochondria, the nuclei were removed by differential centrifugation. K1 and K2 were then centrifuged into a CsCl cushion, thereby concentrating them and separating them from cytoplasmic proteins.

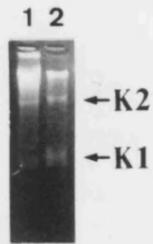


Figure 3.1. High salt precipitation of a *K. lactis* cell lysate.

K. lactis strain IFO1267 was grown in 500 ml YPD to a density of 2×10^8 cells ml^{-1} , and spheroplasted as described in 'Materials and Methods'. The spheroplast pellet was resuspended in 15 ml 50 mM Tris-HCl, 10 mM EDTA, pH 8, containing 1% w/v SDS. 4 ml 5 M NaCl (1 M final concentration) was then added to the lysate, mixed by inverting, and incubated on ice for 1 hr. After centrifugation at 15 K, 4°C, 30 min, in a Sorvall SS-34 rotor, the supernatant was decanted into a clean tube, and the pellet resuspended in 20 ml 50 mM Tris-HCl, 10 mM EDTA, pH 8, containing 1% w/v SDS. 25 μl samples of the resuspended pellet (lane 1) and supernatant (lane 2), were analysed by 0.9% agarose/*in situ* lysis gel electrophoresis.

From the differences between electrophoretic mobilities of pronase E treated and untreated terminal fragments of K1 and K2 DNA prepared using the standard method, it was concluded that the sizes of the TPs are quite small compared to previously characterised TPs of other invertrons (Kikuchi *et al.*, 1984). However, size estimates of 28 and 36 kDa for the K1 and K2 TPs respectively, were obtained from material prepared by the Stam method (Stam *et al.*, 1986). These contradictory results can be explained by the different methods used to isolate the killer plasmids. As discussed above, the standard procedure preferentially selects for plasmids with degraded TPs. Proteolysis might therefore have occurred since SDS also lyses yeast vacuoles that contain many proteases. During isolation of K1 and K2 by the Stam method, the integrity of these organelles is better maintained.

It has also been reported that phenol extraction of a *K. lactis* lysate greatly reduced the yield of killer plasmid DNA (Hishinuma *et al.*, 1984), presumably because the TPs carry the plasmids into the phenol phase. Thus, in trying to isolate K1 and K2, this technique is not appropriate.

3.1.2 Reasons to Scale-Up

The Stam method was described for a small scale (200 ml culture at a density of 2×10^8 cells ml⁻¹) killer plasmid preparation. For the purposes of this study, it was necessary to scale-up this procedure by at least thirty fivefold. For example, to attempt amino-terminal sequencing of the TPs, approximately 0.5 nmol of each TP would be required. Based on the upper assumption of 100 copies of K1 and K2 per haploid cell (Gunge *et al.*, 1982), and assuming 100% recovery, it can be calculated (Figure 3.2) that approximately 7.5 L of culture, at a density of 2×10^8 cells ml⁻¹, must be processed to produce sufficient amounts of protein. Using the lower assumption of 50 copies of the killer plasmids per haploid cell (Gunge *et al.*, 1982), 15 L of culture, at a density of 2×10^8 cells ml⁻¹, must be processed. This represents approximately 1.5 and 2.2 mg of K1 and K2 DNA, respectively. By comparison, assuming a plasmid copy number of 100, a 200 ml culture will only produce 13 pmol of each TP, representing approximately 40 and 60 µg of K1 and K2 DNA, respectively.

The Stam procedure for the isolation of the killer plasmids was demonstrated to separate them from contaminating chromosomal DNA, and to leave their TPs largely intact. It was therefore decided to base a large scale procedure for the purification of K1 and K2 on this method, and a tenfold scale-up to a 2 L culture was attempted (Section 3.2).

Assume 50 copies of K1 and K2 per haploid cell.

∴ 100 copies of each TP per haploid cell.

$100 + N_A = 1.66 \times 10^{-22}$ mol of each TP per haploid cell.

0.5 nmol of each TP is required to attempt amino-terminal sequencing.

(0.5 nmol \approx 14 μ g K1 TP and 18 μ g K2 TP).

mols TP required for sequencing + mols TP per cell = number of cells required.

∴ $0.5 \times 10^{-9} + 1.66 \times 10^{-22} = 3 \times 10^{12}$ cells to produce 0.5 nmol each TP.

Cell density = 2×10^8 cells ml^{-1} .

∴ $3 \times 10^{12} + 2 \times 10^8 = 15$ L culture required to produce 0.5 nmol of each TP, assuming 50 copies of K1 and K2 per haploid cell, a density of 2×10^8 cells ml^{-1} , and 100% recovery of the K1 and K2 TPs.

Similarly, assuming 100 copies of K1 and K2 per haploid cell, a density of 2×10^8 cells ml^{-1} , and 100% recovery of the K1 and K2 TPs, a 7.5 L culture will be required.

Using a similar calculation, and assuming 50 copies of K1 and K2 per haploid cell, a 15 L culture at a density of 2×10^8 cells ml^{-1} will yield approximately 1.5 and 2.2 mg of K1 and K2 DNA, respectively.

Figure 3.2. Calculation of the *K. lactis* culture volume required to produce sufficient amounts of protein to attempt amino-terminal sequencing of the K1 and K2 TPs. N_A (Avogadro constant) = 6.022045×10^{23} mol^{-1} .

3.1.3 A Note on Agarose Gel Electrophoresis of the Killer Plasmids

In the preparation of the maize linear plasmids S-1 and S-2, it was found that the plasmids did not migrate into agarose gels unless first incubated with proteinase K (Kemble and Thompson, 1982). Similarly, K1 and K2 do not migrate unless first treated with protease, or electrophoresed in the presence of SDS. It would appear that the presence of intact TPs inhibit the migration of these invertrons into agarose gels.

Two methods for the direct visualisation of K1 and K2 on agarose gels have been developed. Both methods involve electrophoresis of the plasmids in the presence of SDS. The first, *in situ* lysis agarose gel electrophoresis (Wilson, 1988), involves casting the gel with two parallel sets of wells 0.5 cm apart. 8% w/v SDS in gel loading buffer is added to the trailing well, and the gel run and visualised as normal. With this method, spheroplasts can be loaded into the leading well and lysed *in situ*. The second method, 0.8% agarose/0.2% SDS gel electrophoresis (Stam *et al.*, 1986), involves the inclusion of 0.2% w/v SDS in the gel, running and sample buffers. After running, the gel must first be soaked in water to remove SDS before staining with EtBr. The gel can then be visualised as normal.

Initially, in this study, the *in situ* lysis method was used to visualise the killer plasmids. However, it is the second method which gives the much clearer results. The DNA bands appear sharper with the 0.8% agarose/0.2% SDS gel electrophoresis method. Therefore, where possible, experiments were repeated and samples electrophoresed on 0.8% agarose/0.2% SDS gels.

During the course of this study, samples were also electrophoresed on normal agarose gels to test for the integrity of the terminal proteins, but these gels are not shown. The killer plasmids appear as two distinct bands. Therefore, as normal agarose gels were run in parallel, DNA molecular weight markers were generally not used. Where other DNA species were also visible in *K. lactis* extracts, K1 and K2 are marked.

3.2 ATTEMPT AT SCALE-UP OF THE STAM ISOLATION PROCEDURE TO A 2 LITRE CULTURE

Firstly, it was important to ascertain whether osmotic lysis of a 2 L yeast culture was feasible. A 2 L *K. lactis* YPD culture was grown to a density of 2×10^8 cells ml⁻¹, and spheroplasted as described in 'Materials and Methods'. For reasons discussed in Section 3.3, particular attention was given to the spheroplasting step. Working on ice, the spheroplast pellet was spread evenly around the bottom and along the lower sides of the

centrifuge tube to avoid clumping when the lysis buffer was added. The spheroplasts were then resuspended in 130 ml ice-cold TM buffer (10 mM Tris-HCl, 10 mM MgCl₂, pH 8), containing 1 mM PMSF (phenylmethylsulfonyl fluoride), and left on ice for 5 min. Resuspension was carried out rapidly with vigorous stirring using a glass rod to catch lumps on the side and disperse them. At this stage the lysate was split into two equal halves, and one half homogenised with 10 strokes of a loose-fitting Potter-Elvehjem tissue homogeniser. Cell lysis was followed by light microscopy. The lysates were then centrifuged at 15 K (20,000 × g), 4°C, 15 min, in a Sorvall SS-34 rotor to remove intact cells, cellular debris, nuclei, mitochondria and vacuoles. Samples were taken at each stage and analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 3.3).

The gel shown in Figure 3.3 represents the best achievable results using this method. Chromosomal DNA is visible in the cytoplasmic fraction (lane 3), and use of the Potter-Elvehjem tissue homogeniser further disrupted nuclei (lane 6). In this example, recovery of the killer plasmids was good. The killer plasmids were barely visible in the 15 K pellets (lanes 2 and 5). However, osmotic shock proved to be unreliable for the lysis of 2 L cultures. Sometimes, lysis, as judged by agarose gel electrophoresis and light microscopy, was very poor, or the 15 K supernatant contained a greater amount of contaminating chromosomal DNA as shown in Figure 3.3. This happened despite great care being taken in the growth and spheroplasting of the yeast cells. Decreasing the volume of the lysis buffer resulted in reduced spheroplast lysis, and no reduction in the proportion of contaminating chromosomal DNA.

Low pH values decrease the resistance of spheroplasts to osmotic shock (Indge, 1968), and pH was therefore not varied in this experiment. The failure of this method to be reliable for large yeast cultures is probably due to the size of the spheroplast pellet. A small spheroplast pellet has a higher surface area to mass ratio compared with a larger pellet, and so the spheroplasts will tend to clump less and disperse more quickly and evenly into the lysis buffer. Spreading the large spheroplast pellet around the bottom and sides of the centrifuge tube did not wholly solve the problem of clumping and uneven dispersion.

The purpose of this study was to develop a reliable, large scale, procedure for the purification of the *K. lactis* linear killer plasmids. A reliable lysis method, which preferably separates chromosomal DNA from the killer plasmids at this first step, is necessary. Osmotic shock has been demonstrated to be an unsuitable method of lysis when attempted on a 2 L culture of *K. lactis* strain IFO1267.

When following the Stam procedure, if any chromosomal DNA remains in the supernatant after the 15 K spin, it will be concentrated, along with the killer plasmids, when spun into a CsCl cushion. This is demonstrated in Figure 3.4, where 13 ml of a 15 K supernatant,

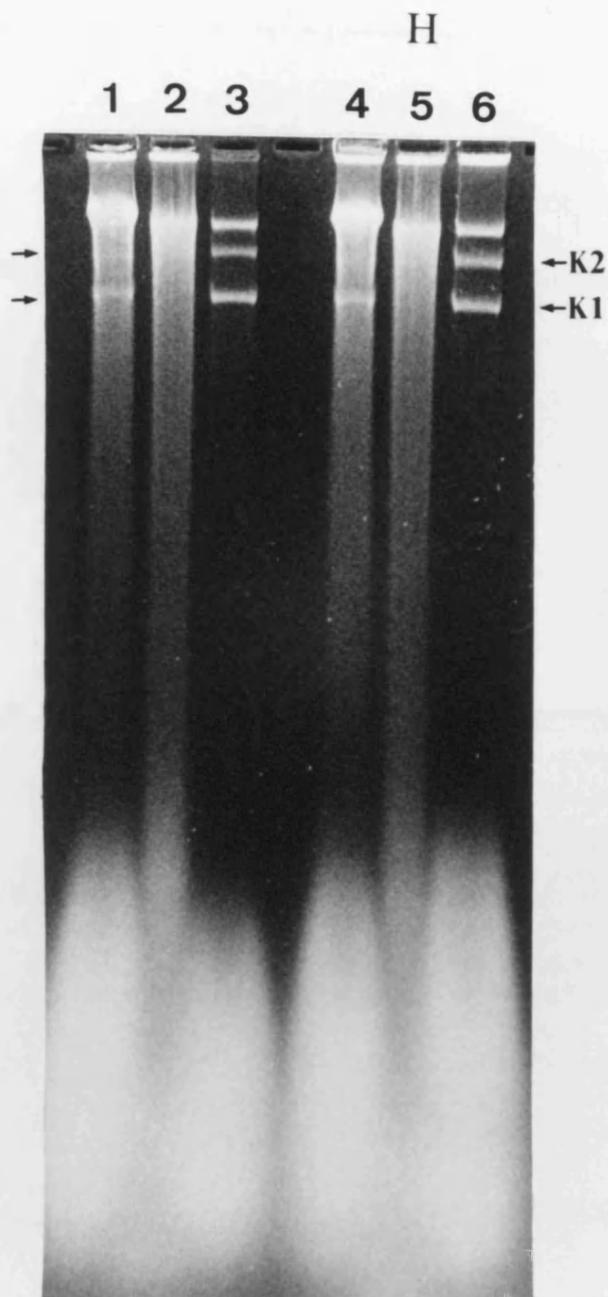


Figure 3.3. Osmotic shock treatment of *K. lactis* spheroplasts prepared from a 2 L YPD culture at a density of 2×10^8 cells ml^{-1} .

Spheroplasts were prepared and lysed as described in Section 3.2. The lysate was split into two equal halves, and one half (H, lanes 4 to 6) treated with 10 strokes of a loose-fitting Potter-Elvehjem tissue homogeniser. After centrifugation, the supernatants were decanted into clean tubes, and the pellets vigorously resuspended in 65 ml ice-cold TM buffer containing 1 mM PMSF and 0.2% w/v SDS. 25 μl samples of the cell lysates (lanes 1 and 4), 15 K pellets (lanes 2 and 5), and 15 K supernatants (lanes 3 and 6), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

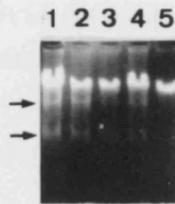


Figure 3.4. The Stam, *et al.* (1986) killer plasmid isolation procedure scaled up to a 2 L YPD *K. lactis* culture at a density of 2×10^8 cells ml^{-1} .

Spheroplasts were prepared and lysed as described in Section 3.2. 13 ml of the 15 K supernatant was loaded onto a 1 ml 40% w/v CsCl cushion in TM buffer, and spun at 40 K, 10°C, 90 min, in a Sorvall TST41.14 rotor. After centrifugation, the zones were fractionated using a Pasteur pipette, and the pellet resuspended in 1 ml TM buffer containing 0.2% w/v SDS. 20 μl of the 15 K (lane 1) and 40 K (lane 2) supernatants, 1.5 μl (lane 3) and 3 μl (lane 4) of the CsCl fraction, and 1.5 μl of the 40 K pellet (lane 5), were analysed by 0.9% agarose/*in situ* lysis gel electrophoresis. Arrows indicate position of killer plasmids.

contaminated with chromosomal DNA, has been loaded onto a 1 ml 40% w/v CsCl cushion in TM buffer, and spun at 40 K, 10°C, 90 min, in a Sorvall TST41.14 rotor. Chromosomal DNA has clearly entered the CsCl cushion along with the killer plasmids (lanes 3 and 4). Also, in this example, a large proportion of the killer plasmids did not enter the CsCl cushion (see also Section 3.4.1).

Not only have these initial experiments demonstrated the need to examine other lysis methods for yeast spheroplasts, but they have also demonstrated that other methods for the removal of cytoplasmic contaminants will have to be investigated. The Stam procedure does not appear to be amenable to scale-up. Furthermore, after spinning into a CsCl cushion, Stam, *et al.* purified the killer plasmids on preparative 0.9% agarose/0.2% SDS gels. Even with a 200 ml culture, the CsCl step does not effectively remove contaminating cytoplasmic proteins, and preparative gel electrophoresis is not feasible on a large scale. Sections 3.3 and 3.4 therefore examine other lysis and isolation methods for the large scale purification of the killer plasmids.

3.3 LYSIS METHODS

The first consideration in the isolation of the killer plasmids is that yeast nuclei, and preferably also the vacuoles, must be kept largely intact. As already discussed (Section 3.1.1), yeast vacuoles contain many proteases which may degrade the TPs, and it is advantageous to remove contaminating chromosomal DNA at this first step. The considerations in the isolation of the killer plasmids are therefore similar to the isolation of yeast nuclei, except that it is the cytoplasmic fraction that is required.

Yeast nuclei have been considered difficult to isolate in comparison to animal cell systems. One reason for this is because yeast have a cell wall, and probably the single most crucial step in yeast nuclear isolation is the spheroplasting step. Hence, particular attention was devoted to this step in the isolation of the killer plasmids. Throughout this work, good killer plasmid preparations were never obtained from poorly spheroplasted cells. For a detailed spheroplasting protocol see 'Materials and Methods'.

It is also possible to isolate nuclei from normal yeast cells using an Eaton press (Duffus, 1969; Bhargava and Halvorson, 1971), a modified French press (Bhargava and Halvorson, 1971; Sajdel-Sulkowska *et al.*, 1974), or a Biotic X-Press (Wintersberger *et al.*, 1973). These methods have the advantage that the cells, and consequently the nuclei, are initially in a normal state, but they have the disadvantages that yields are variable and that nuclei may suffer considerable damage. Therefore, because of these disadvantages, these methods were considered unsuitable for the isolation of the killer plasmids.

Lysis methods employed in the isolation of nuclei from the commonly used animal cell systems, and in the isolation of yeast vacuoles, may also be considered as suitable methods for the first step in the purification of the killer plasmids. As with the isolation of yeast nuclei, the methods rely on gentle cell lysis which retain the nuclear and vacuolar membranes intact.

Three lysis methods, all based on the prior removal of the yeast cell wall, were chosen as suitable methods for study in the isolation of the killer plasmids. The three methods are based on Ficoll, DEAE-dextran, and Nonidet P40 (NP40) as the lysing agents. Spheroplasts were prepared from 2 L *K. lactis* YPD cultures as described in 'Materials and Methods'.

3.3.1 Ficoll

The method of Wintersberger *et al.* (1973) provides the most consistently successful methodology for the isolation of yeast nuclei. The essential feature of the approach is the discovery that 18% Ficoll in hypotonic buffer lyses spheroplasts but does not lyse nuclei. The use of Ficoll in hypotonic buffer also acts to prevent lysis of vacuoles, which reduces proteolysis during purification. The following protocol is based on the method of Lohr and Ide (1979).

Working on ice, the spheroplast pellet was spread evenly around the bottom and along the lower sides of the centrifuge tube, and then resuspended in 50 ml of 18 % Ficoll, 20 mM KH_2PO_4 , 0.5 mM Ca^{2+} , pH 6.5, containing 1 mM PMSF. Resuspension was carried out rapidly with vigorous stirring using a glass rod. At this stage the lysate was split into two halves, and one half homogenised with 10 strokes of a loose-fitting Potter-Elvehjem tissue homogeniser. Cell lysis was followed by light microscopy. The lysates were then centrifuged at 15 K, 4°C, 15 min, in a Sorvall SS-34 rotor. Samples were taken at each stage and analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 3.5).

As shown in Figure 3.5, similar results were observed with Ficoll-induced lysis as with osmotic shock. Chromosomal DNA was again visible in the cytoplasmic fraction (lane 3), and use of the Potter-Elvehjem tissue homogeniser further disrupted nuclei (lane 6). From the proportion of killer plasmids in the 15 K pellets (lanes 2 and 5) compared to that in the 15 K supernatants (lanes 3 and 6), and as judged by light microscopy, cell lysis was not complete using this method.

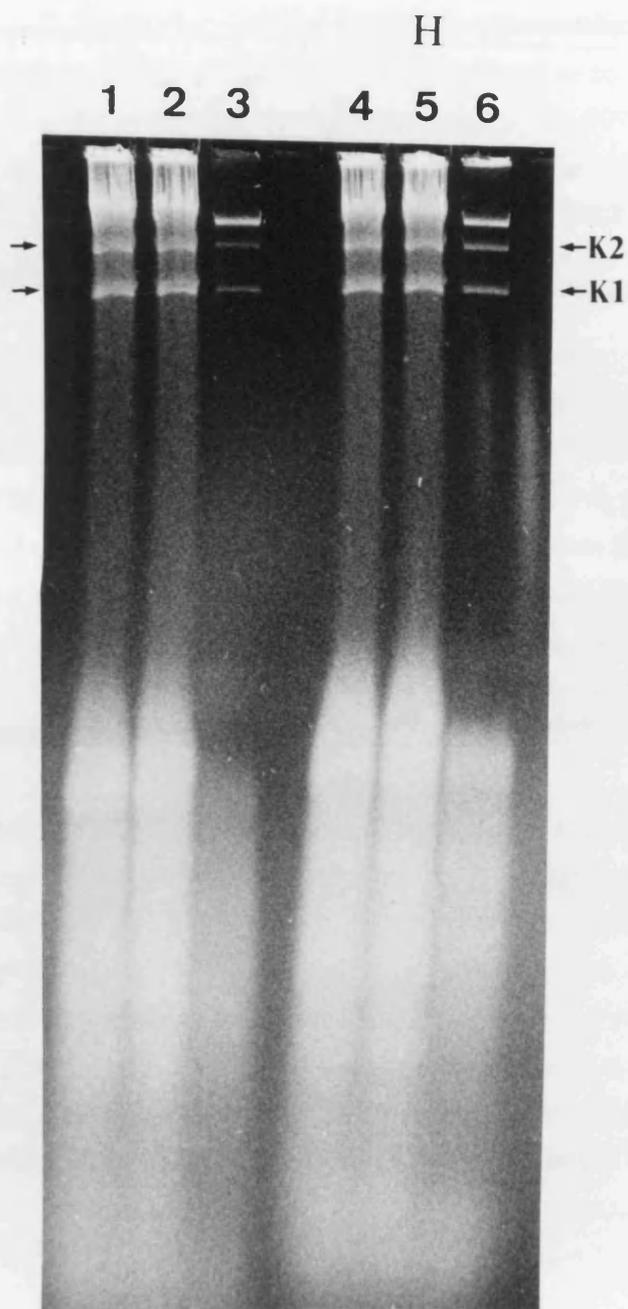


Figure 3.5. Ficoll-induced lysis of *K. lactis* spheroplasts.

Spheroplasts were prepared and lysed as described in Section 3.3.1. The lysate was split into two equal halves, and one half (H, lanes 4 to 6) treated with 10 strokes of a loose-fitting Potter-Elvehjem tissue homogeniser. After centrifugation, the supernatants were decanted into clean tubes, and the pellets vigorously resuspended in 25 ml of the lysis buffer containing 0.2% w/v SDS. 15 μ l samples of the cell lysates (lanes 1 and 4), 15 K pellets (lanes 2 and 5), and 15 K supernatants (lanes 3 and 6), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

3.3.2 DEAE-Dextran

DEAE-dextran (diethylaminoethyl-dextran) is a polybasic macromolecule of molecular weight 500 000. This high molecular weight polybase was found to be ideally suited for lysis of yeast spheroplasts while preserving the vacuoles (Dürr, *et al.*, 1975) Small doses of DEAE-dextran rapidly and completely adsorbed to the surface of the spheroplasts at 0°C, and lysis was initiated by increasing the temperature to 30°C for a short time. The treatment disrupted more than 99% of the spheroplasts and left at least 70% of the vacuoles intact.

DEAE-dextran appears to have specificity of attack. This is probably due to the separation in time of adsorption and lysis. The vacuoles are not accessible to DEAE-dextran at the time of adsorption. When lysis occurs, following adsorption, it is assumed that the liberated vacuoles do not come into contact with any free polybase as this persists firmly adsorbed to the disrupted cell membrane. A second reason is that any free cationic groups are probably neutralised by released cytoplasmic polyanions. Comparative studies with spheroplasts and isolated vacuoles also revealed that the plasmalemma is more sensitive to DEAE-dextran than the tonoplast (Dürr, *et al.*, 1975).

The standard procedure of DEAE-dextran induced lysis for the preparation of yeast vacuoles involves adding 5-10 µg of DEAE-dextran per ml to a suspension containing 10⁸ spheroplasts. A 2 L culture at 2×10⁸ cells ml⁻¹ will yield 4×10¹¹ cells. Following this protocol would require resuspending the spheroplasts from this size culture in 4 L of buffer. Such a volume is not practical with regard to raising the temperature from 0 to 30°C to initiate lysis, is too large for differential centrifugation in a Sorvall SS-34 or similar rotor, does not allow for rapid work, and restricts the choices for the next purification step. For this method to be of use in this instance, it would have to work in a much smaller volume. Spheroplasts obtained from a 2 L culture were therefore resuspended in 130 ml of buffer. Otherwise, the following protocol is based on the method of Dürr, *et al.* (1975).

Working on ice, the spheroplast pellet was spread evenly around the bottom and along the lower sides of the centrifuge tube, resuspended in 130 ml 0.7 M sorbitol buffered with 5 mM MOPS, pH 6, and 40 mg of DEAE-dextran added to the suspension. The spheroplasts were allowed to adsorb the polybase for 1 min, and then incubated for 6 min at 30°C. Cell lysis was followed by light microscopy. The lysate was then centrifuged at 15 K, 4°C, 15 min, in a Sorvall SS-34 rotor. Samples were taken at each stage and analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 3.6).

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DEAE-dextran (diethylaminoethyl-dextran) is a polybasic macromolecule of molecular weight 500 000. This high molecular weight polybase was found to be ideally suited for the lysis of yeast spheroplasts while preserving the vacuoles (Dürr *et al.*, 1975). Small doses of DEAE-dextran rapidly and completely adsorbed to the surface of the spheroplasts at 0°C, and lysis was initiated by increasing the temperature to 30°C for a short time. The treatment disrupted more than 99% of the spheroplasts and left at least 70% of the vacuoles intact.

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The standard procedure of DEAE-dextran induced lysis for the preparation of yeast vacuoles involves adding 5-10 µg of DEAE-dextran per ml of suspension containing 10^8 spheroplasts. A 2 L culture at 2×10^8 cells ml⁻¹ will yield 4×10^{11} cells. Following this protocol would require resuspending the spheroplasts from this size culture in 4 L of buffer. Such a volume is not practical with regard to raising the temperature from 0 to 30°C to initiate lysis, is too large for differential centrifugation in a Sorvall SS-34 or similar rotor, does not allow for rapid work, and restricts the choices for the next purification step. For this method to be of use in this instance, it would have to work in a much smaller volume. Spheroplasts obtained from a 2 L culture were therefore resuspended in 130 ml of buffer. Otherwise, the following protocol is based on the method of Dürr *et al.* (1975).

Working on ice, the spheroplast pellet was spread evenly around the bottom and along the lower sides of the centrifuge tube, resuspended in 130 ml 0.7 M sorbitol buffered with 5 mM MOPS, pH 6, and 40 mg of DEAE-dextran added to the suspension. The spheroplasts were allowed to adsorb the polybase for 1 min, and then incubated for 6 min at 30°C. Cell lysis was followed by light microscopy. The lysate was then centrifuged at 15 K, 4°C, 15 min, in a Sorvall SS-34 rotor. Samples were taken at each stage and analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 3.6).



Figure 3.6. DEAE-dextran-induced lysis of *K. lactis* spheroplasts.

Spheroplasts were prepared and lysed as described in Section 3.3.2. After centrifugation, the supernatant was decanted into a clean tube, and the pellet vigorously resuspended in 130 ml of the lysis buffer containing 0.2% w/v SDS. 25 μ l samples of the cell lysate (lane 1), 15 K pellet (lane 2), and 15 K supernatant (lane 3), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

As judged by light microscopy, and as shown by agarose gel electrophoresis (Figure 3.6), spheroplast lysis was very poor using this method. A small amount of high molecular weight DNA was visible in the 15 K supernatant (lane 3), but no killer plasmids were visible. The killer plasmids and chromosomal DNA were only visible in the 15 K pellet (lane 2). Increasing the time of adsorption from 1 to 5 min, increasing the time of incubation at 30°C from 6 to 15 min, or increasing the amount of DEAE-dextran added to the spheroplast suspension tenfold, did not increase spheroplast lysis.

3.3.3 Nonidet P40 (NP40)

Numerous procedures for preparing separated nuclear and cytoplasmic fractions of mammalian cells are described in the literature (Busch and Daskal, 1977; Goodwin, 1989). The first step is the removal of as much of the cytoplasm from cell nuclei as possible. The most commonly used method is to allow the cells to swell in hypotonic buffer followed by homogenisation with between five and ten strokes in a Dounce or Potter-Elvehjem tissue homogeniser. Following centrifugation and resuspension, a mixed detergent solution, composed of non-ionic Tween 40 and anionic sodium deoxycholate, can be added to the nuclei to strip the remaining cytoplasm and the outer nuclear membrane (Traub *et al.*, 1964). The choice of non-ionic detergent is not critical, and Tween 80 and NP40 can be substituted (Penman, 1969).

In a modified technique, the initial fractionation into cytoplasm and crude nuclei is accomplished with a nonionic detergent (Borun *et al.*, 1967). This technique of fractionating with detergent alone consists of swelling washed cells in buffer containing 0.5% v/v NP40. The following protocol, using NP40 to lyse the *K. lactis* spheroplasts, is based on the method of Borun *et al.* (1967).

Working on ice, the spheroplast pellet was spread evenly around the bottom and along the lower sides of the centrifuge tube, and resuspended in 50 ml 20 mM Tris-HCl, 100 mM NaCl, 1mM EDTA, pH 7.5, containing 1 mM PMSF. A 5% v/v solution of NP40 was then added drop wise to a final concentration of 0.5%, and the suspension was stirred gently for 10 min using a glass rod. Cell lysis was followed by light microscopy. The lysate was then centrifuged at 15 K, 4°C, 15 min, in a Sorvall SS-34 rotor. Samples were taken at each stage and analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 3.7).

As judged by light microscopy, lysis was very good using this method. No unlysed spheroplasts were visualised. The gel shown in Figure 3.7 shows a typical result using this method. Very little high molecular weight DNA was visible in the 15 K supernatant (lane 3), whereas the killer plasmids were clearly visible in this fraction. The chromosomal

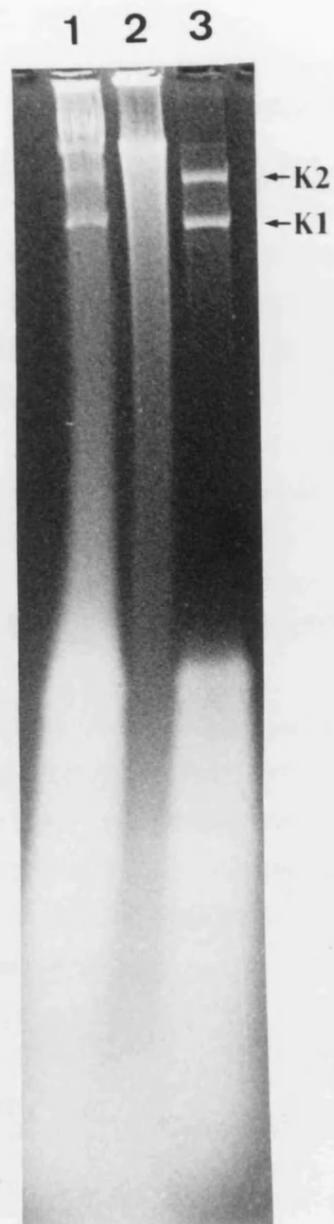


Figure 3.7. NP40-induced lysis of *K. lactis* spheroplasts.

Spheroplasts were prepared and lysed as described in Section 3.3.3. After centrifugation, the supernatant was decanted into a clean tube, and the pellet vigorously resuspended in 50 ml of the lysis buffer containing 0.2% w/v SDS. 15 μ l samples of the cell lysate (lane 1), 15 K pellet (lane 2), and 15 K supernatant (lane 3), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

DNA was found in the 15 K pellet (lane 2), but the killer plasmids were barely visible in this fraction. This method proved to be highly reproducible.

3.4 REMOVAL OF CYTOPLASMIC PROTEINS

Not only did the attempt at scale-up of the Stam isolation procedure for the killer plasmids (Section 3.2) demonstrate the need to examine other spheroplast lysis methods, but it also demonstrated the necessity to investigate other purification procedures following the preparation of the cytoplasmic extract. Centrifugation of the killer plasmids into a 40% w/v CsCl cushion did not prove to be a reproducible procedure. Figure 3.4 clearly demonstrates that a large proportion of the killer plasmids are still present in the sample zone loaded onto the CsCl cushion (lane 2).

Two purification procedures were chosen for investigation as alternatives to the Stam CsCl cushion method. Firstly, it was attempted to improve the Stam *et al.* (1986) method by using CsCl step gradients. Secondly, an entirely new approach was taken by attempting to purify the killer plasmids by ion exchange chromatography.

Spheroplast lysis using 2% w/v sarkosyl (*N*-Dodecanoylsarcosinate, sodium salt) was the method chosen for these experiments. Although nuclei are also lysed, the method is highly reproducible, and no other reliable lysis method had been developed at the time in this study. These experiments are comparisons of the purification procedures, and therefore the method of lysis is largely unimportant. Spheroplasts were prepared from 2 L *K. lactis* YPD cultures as described in 'Materials and Methods'.

3.4.1 Centrifugation on CsCl Step Gradients

Both K1 and K2 have identical buoyant densities of 1.687 g cm^{-3} . Centrifugation for a longer time than used in the Stam *et al.* (1986) method would therefore eventually pellet the killer plasmids, as a 40% w/v CsCl cushion corresponds to a density of approximately 1.3 g cm^{-3} . This was demonstrated by spinning for 10 hours under the same conditions (40 K, 10°C , in a Sorvall TST41.14 rotor). After this time, all of the killer plasmid, and high molecular weight DNA, had pelleted (results not shown). The CsCl concentration used in the Stam *et al.* (1986) method is also somewhat surprising as proteins have a buoyant density of approximately 1.3 g cm^{-3} in CsCl gradients. It is therefore possible that protein could enter a 40% w/v CsCl cushion. As the mass of the TPs is very small compared to the mass of DNA, it is unlikely that the TPs would inhibit the entry of the killer plasmids into a CsCl cushion of higher density.

The following experiment attempted to overcome these problems by centrifuging the killer plasmids into a CsCl step gradient of $\rho = 1.5825 \text{ g cm}^{-3}$ and $\rho = 1.7846 \text{ g cm}^{-3}$. The killer plasmids would be expected to become trapped in the $\rho = 1.5825 \text{ g cm}^{-3}$ zone; their buoyant densities being too low to enter the $\rho = 1.7846 \text{ g cm}^{-3}$ zone, and proteins should not enter the $\rho = 1.5825 \text{ g cm}^{-3}$ zone. Longer centrifugation times should therefore be possible.

Working on ice, the spheroplast pellet was resuspended in 50 ml 10 mM Tris-HCl, 10 mM EDTA, pH 8, containing 1 mM PMSF, and sarkosyl added to 2% w/v. Cell lysis was followed by light microscopy, and the lysate centrifuged at 15 K, 4°C, 15 min, in a Sorvall SS-34 rotor. The supernatant was split into two and each layered onto a 3 ml $\rho = 1.7846 \text{ g cm}^{-3}$ (107.08% w/v)/4 ml $\rho = 1.5825 \text{ g cm}^{-3}$ (79.13% w/v) CsCl step gradients in 10 mM Tris-HCl, 10 mM EDTA, pH 8, and then centrifuged at 25 K, 18 hr, 10°C, in a Sorvall AH627 rotor. After centrifugation, the zones (sample, $\rho = 1.5825 \text{ g cm}^{-3}$ and $\rho = 1.7846 \text{ g cm}^{-3}$) were carefully recovered using a Pasteur pipette and pooled. The 25 K pellets were vigorously resuspended in 2 ml of the lysis buffer containing 1 mM PMSF and 2% w/v sarkosyl. Samples were taken at each stage and analysed by 0.9% agarose/*in situ* lysis gel electrophoresis (Figure 3.8).

In the example shown in Figure 3.8, the killer plasmids, and high molecular weight DNA, were visible in both the $\rho = 1.5825 \text{ g cm}^{-3}$ and $\rho = 1.7846 \text{ g cm}^{-3}$ zones (lanes 5-8). No DNA was visible in the sample zone, and only a small amount of DNA was visible in the 25 K pellet. However, the result was not entirely reproducible, as sometimes DNA was visible in the sample zone. Generally, no DNA was visible in the 25 K pellet, DNA was always found in both the $\rho = 1.5825 \text{ g cm}^{-3}$ and $\rho = 1.7846 \text{ g cm}^{-3}$ zones, and recovery of K1 and K2 was improved upon the Stam *et al.* (1986) method.

The experiment was also performed on 50/60% w/v ($\rho \approx 1.37$ and 1.45 g cm^{-3} , respectively) CsCl step gradients, by centrifuging at 40 K, 2-5 hrs, 10°C, in a Sorvall TST41.14 rotor. However, no improvement of the Stam *et al.* (1986) procedure was obtained. Either only a small amount of the killer plasmids entered the CsCl with the shorter spin times, or they began to pellet and were found in all of the zones with the longer spin times (results not shown).

3.4.2 Anion Exchange Chromatography

Ion exchange chromatography is a very powerful tool in biochemical purification, separating molecules according to differences in charge. DE52 (Whatman) is a pre-swollen, microgranular, DEAE-cellulose (diethylaminoethyl-cellulose) resin. It is a weak

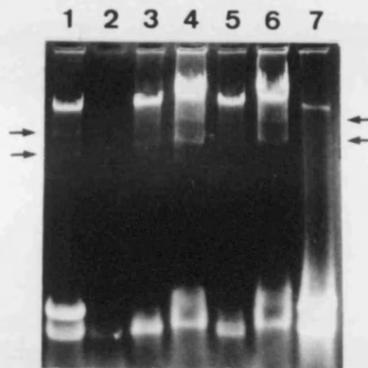


Figure 3.8. Centrifugation of the killer plasmids on a CsCl step gradient.

Preparation of the sample, and experimental conditions, were as described in Section 3.4.1. 25 μ l of the 15 K supernatant (lane 1), 25 μ l of the 25 K supernatant (lane 2), 4 μ l (lane 3) and 25 μ l (lane 4) of the $\rho = 1.5825$ zone, 3 μ l (lane 5) and 25 μ l (lane 6) of the $\rho = 1.7846$ zone, and 1 μ l of the 25 K pellet (lane 7), were analysed by 0.9% agarose/*in situ* lysis gel electrophoresis. Arrows indicate position of killer plasmids.

anion exchanger which exhibits good resolution with good flow rates, and is commonly used in the separation of biopolymers with low to high negative charges. The basis of this separation is therefore the interaction of the negatively charged phosphate groups along the DNA backbone of the killer plasmids with the positively charged DE52 matrix.

As no information could be found in the literature for the separation of nucleic acid using DE52, the choice of column running conditions used in the following protocol were based on the high performance separation of plasmid DNA (pBR322), and DNA restriction fragments, on a Pharmacia Mono Q[®] HR column (Pharmacia, Laboratory Separation Division, Pharmacia FPLC[®]: Application File, AF19 50-01-343; Pharmacia LKB Biotechnology, Application Note: FPLC[®], FPDA 50-01-478). Mono Q[®] (MonoBeads[™] linked to quaternary amine groups) is a strong anion exchanger, designed for the Pharmacia FPLC[®] system. The principles for this separation are the same as for the attempt to use DE52 to purify the killer plasmids, and therefore the conditions could be used as a guide.

A 15 K supernatant (approximately 25 ml) was prepared as described in section 3.4.1, and dialysed against three changes of 500 ml 20 mM Tris-HCl, 1 mM EDTA, pH 8, at 1 hourly intervals, at 4°C, before loading onto a 15 ml, 1 cm diameter, DE52 column, previously equilibrated with 20 mM Tris-HCl, 1 mM EDTA, pH 8, at a flow rate of approximately 0.5 ml min⁻¹. 15 ml 20 mM Tris-HCl, 350 mM NaCl, 1 mM EDTA, pH 8 was then applied to the column at the the same flow rate, followed by 15 ml 20 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, pH 8. Running buffers were kept on ice. 5 ml fractions were collected, and samples of each analysed by 0.9% agarose/*in situ* lysis gel electrophoresis (Figure 3.9).

Figure 3.9 shows the nucleic acid elution profile of the 15 ml DE52 column, as visualised by 0.9% agarose/*in situ* lysis gel electrophoresis. The killer plasmids, along with high molecular weight DNA, clearly bound to the DE52 matrix, and were eluted by 1 M NaCl, but not by 350 mM NaCl. DNA was only visible in fractions 9 and 10. By comparison of the sample loaded (lane 1) and fractions 9 and 10, recovery of K1 and K2 appeared to be very good. Also, the killer plasmids were concentrated from 25 ml, down to a maximum of 10 ml.

There was a large decrease in the total bed volume of the DE52 column, of approximately one third of its original volume, upon loading of the sample. This may have be due to overloading the column, too high a salt concentration in the form of sarkosyl and NaCl, or both.

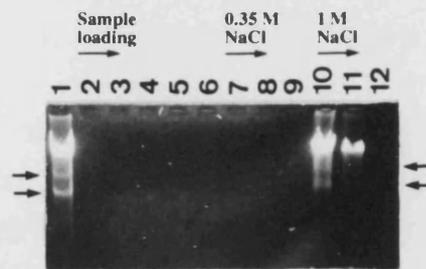


Figure 3.9. Chromatography of *K. lactis* nucleic acid on a 15 ml DE52 column.

Preparation of the sample, and column running conditions, were as described in Section 3.4.2. 25 μ l of the 15 K supernatant (lane 1), and 5 μ l of each fraction (lanes 2 to 12) were analysed by 0.9% agarose/*in situ* lysis gel electrophoresis. Fractions 1 to 5 (lanes 2 to 6) represent sample loading, fractions 6 to 8 (lanes 7 to 9) application of 350 mM NaCl, and fractions 9 to 11 (lanes 10 to 12) application of 1 M NaCl. Arrows indicate position of killer plasmids.

3.5 CONCLUSION

The failure of the Stam isolation procedure for K1 and K2 to lend itself to scale-up led to the investigation of other lysis methods for *K. lactis* spheroplasts, and other methods for the purification of the killer plasmids following the preparation of the cytoplasmic fraction. Three lysis methods were investigated using Ficoll, DEAE-dextran, and NP40 as the lysing agents. Ficoll-induced lysis gave results similar to that obtained using osmotic shock, and therefore had no obvious advantages over this method other than the lysis step could be performed in a smaller volume and gave consistent, albeit poor, results. High molecular weight DNA was visible in the cytoplasmic fraction, and lysis was not complete using this method. Comparison of the killer plasmids in lanes 2 and 3, and 5 and 6, of Figure 3.5, would suggest that the recovery of K1 and K2 was 50% or less.

DEAE-dextran-induced lysis gave very poor results. Very little lysis was observed as judged by light microscopy or by agarose gel electrophoresis. The method is obviously not suited for the lysis of large numbers of spheroplasts at high density. It is known that the adsorption of the polybase occurs very rapidly (Dürr *et al.*, 1975). Therefore, it is possible that at high spheroplast densities only a small proportion of spheroplasts adsorb the polybase. Lower spheroplast densities are probably required to ensure an even distribution of the polybase over the spheroplast population or individual spheroplasts. Also, at high spheroplast densities, the charge environment may be different and could therefore effect the adsorption of DEAE-dextran to the plasmalemma.

NP40-induced spheroplast lysis gave much better results than osmotic shock. As judged by light microscopy, 100% lysis was achieved. By comparison of the proportion of killer plasmids in the 15 K pellet and the 15 K supernatant (Figure 3.7, lanes 2 and 3), almost 100% recovery of K1 and K2 was obtained. Nuclei appear to have remained largely intact as high molecular weight DNA is barely visible in the cytoplasmic fraction. Generally, no high molecular weight DNA was visible. The method was highly reproducible for spheroplasts produced from both small and large cultures. The method was tested for culture volumes of up to 4 L and the same results obtained.

As already discussed, for the purpose of this study, a reliable procedure for the lysis of *K. lactis* spheroplasts that removes high molecular weight DNA at the first step is required. Spheroplast lysis with 0.5% v/v NP40 meets these criterion, but it is unlikely that this method retains vacuoles intact. However, working rapidly on ice, with the inclusion of protease inhibitors, would minimise the effect of vacuolar lysis. Furthermore, even the best reported method of spheroplast lysis for the preparation of yeast vacuoles using DEAE-dextran reported a maximum of 30% degraded vacuoles when greater than 99% spheroplast lysis was achieved (Dürr *et al.*, 1975). With any lysis method, vacuolar lysis is

inevitable. Taking this into consideration, the advantages of NP40-induced lysis over the other methods investigated were considerable, and the method was subsequently used as the first step for a large scale isolation procedure for the killer plasmids. As will be discussed in 'Chapter 6', the choice of NP40 as the lysing agent was justified as no apparent degradation of the TPs was observed in iodination experiments or by gel shift assays.

In the CsCl step gradient and the DE52 column experiments, only the behaviour of the DNA was followed. At this stage, the behaviour of protein was not considered. However, from the colour of fractions, and the A_{280} profile of the DE52 column run (results not shown, but refer to Section 4.2 and 'Appendix I'), good separation of killer plasmids from other cellular components had clearly been obtained with both methods.

Centrifugation of the killer plasmids into a $\rho = 1.5825/1.7846 \text{ g cm}^{-3}$ step gradient gave some improvement over use of a 40% w/v CsCl cushion. Combined with NP40-induced lysis, this method would probably give results as good as reported by Stam *et al.* (1986), but does, however, have the drawback of necessitating a long spin time. To help maintain the integrity of the TPs, a more rapid method of separation was desirable. That DNA was found in the $\rho = 1.7846 \text{ g cm}^{-3}$ zone, as well as the $\rho = 1.5825 \text{ g cm}^{-3}$ zone, was probably because DNA bands at the boundary of the two zones, and was therefore visible in both zones upon fractionation. Alternatively, some mixing of the two CsCl zones could have occurred during gradient formation or centrifugation.

The CsCl step gradient could probably be further improved by the addition of a third CsCl zone of, for example, $\rho = 1.4 \text{ g cm}^{-3}$. This would prevent mixing of the sample zone, and therefore cytoplasmic proteins, with the $\rho = 1.5825$ and 1.7846 g cm^{-3} zones upon fractionation.

The results obtained with the DE52 column run were promising. The killer plasmids bound to the column, were not eluted with 350 mM NaCl, but were eluted with 1 M NaCl. Furthermore, the sample only took 50 min to load, and the run was complete in less than 2 hours. For this reason, this method has significant advantages over CsCl step gradients. Also, recovery of DNA from the DE52 column run was good.

The Stam *et al.* (1986) and the CsCl step gradient methods were tested extensively. Although the CsCl step gradient was an improvement on the Stam *et al.* (1986) method, it was considered that any further improvements would not be significant, and the method was still not entirely reliable. In comparison, the results obtained with the DE52 column suggested that significant improvements could be made. For this reason, and the rapidity of the method over CsCl step gradients, the purification of the killer plasmids by DE52

column chromatography was investigated further in preference to purification on CsCl step gradients.

CHAPTER 4

DEVELOPMENT OF A LARGE SCALE KILLER PLASMID PURIFICATION PROCEDURE

4.1 INTRODUCTION

This chapter discusses the development of DE52 column chromatography for large scale purification of the killer plasmids, and methods for their further purification. NP40 was used as the lysing agent in the following experiments.

4.2 DE52 COLUMN CHROMATOGRAPHY

At low ionic strengths, competition for charged groups on the DE52 resin is at a minimum and substances are bound strongly. Increasing the ionic strength increases competition and reduces the interaction between the anion exchanger and the sample substances, resulting in their elution. The various components in the sample have different affinities for the DE52 resin, and so variations in the pH and ionic strength of the eluent will cause their elution at different times, and thus their separation from each other. Continuous or stepwise ionic strength gradients can be used to achieve this.

Continuous ionic strength gradients are easy to prepare and very reproducible. Two buffers of differing ionic strength are mixed together, and if the volume ratio is changed linearly, the ionic strength changes linearly. Stepwise ionic strength gradients are produced by the sequential use of the same buffer at different ionic strengths. Stepwise elution is technically more simple, but has its disadvantages as substances eluted by a sharp change in ionic strength tend to elute close together. Peaks, therefore, have sharp fronts and pronounced tailing since they contain more than one component. However, with stepwise gradients, a substance will elute in a smaller volume than for the same substance eluted with a continuous gradient. This was considered advantageous in the purification of K1 and K2 as a second purification step was anticipated. It was expected that the killer plasmids would bind much more strongly than protein to the DE52 resin, and so the advantages of continuous ionic strength gradients were not considered significant.

The following two experiments (Sections 4.2.1 and 4.2.2) were designed to ascertain whether or not a stepwise gradient would be suitable in the purification of the killer

plasmids on a DE52 column. These data were then used to scale-up the procedure. The columns used were packed as described in 'Materials and Methods'.

4.2.1 Application of a Continuous Ionic Strength Gradient to a 15 ml DE52 column

Firstly, it was necessary to examine accurately the behaviour of the killer plasmids on a DE52 column. This was achieved by running a continuous linear NaCl gradient, after application of the cytoplasmic fraction, to determine the salt concentration at which the killer plasmids elute. These data could then be used to develop a step gradient protocol.

It had already been determined that K1 and K2 are eluted by 1 M NaCl, but not by 350 mM NaCl. As lysis was performed in the presence of 100 mM NaCl, the gradient chosen for this experiment was from 100 mM NaCl to 1 M NaCl over a 50 ml gradient volume.

Spheroplasts were prepared from a 1 L *K. lactis* YPD culture as described in 'Materials and Methods', and lysed as described in Section 3.3.3. The 15 K supernatant (approximately 20 ml) was loaded onto a 15 ml, 1 cm diameter, DE52 column, previously equilibrated with 0.1 M NTE (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.5), at a flow rate of approximately 0.5 ml min⁻¹. A 50 ml linear gradient of increasing NaCl concentration from 100 mM to 1 M, in 20 mM Tris, 1 mM EDTA, pH 7.5, was then applied using a home made gradient mixer (School of Biology Workshop, University of Leicester), at a flow rate of approximately 0.5 ml min⁻¹. The equilibration buffer was kept on ice, and the gradient buffers were kept ice-cold prior to application of the gradient. Fractions (5 ml) were monitored by absorbance at 280 nm (Figure 4.1), and samples of each analysed by 0.9% agarose/*in situ* lysis gel electrophoresis (Figure 4.2).

The gradient was stopped after 45 ml, and a total of 13 fractions had been collected. Figure 4.2 shows the nucleic acid elution profile of the 15 ml DE52 column, as visualised by 0.9% agarose/*in situ* lysis gel electrophoresis. K1 and K2 were found in fractions 10 to 12, and were concentrated from 20 ml, down to a maximum of 15 ml. The majority of the killer plasmids were found in fraction 11, corresponding to peak 4 of the elution profile (Figure 4.1).

It was thus shown that K1 and K2 start to elute at between 550 and 640 mM NaCl under these conditions (Figure 4.1). Note, however, that these figures do not take into account the void volume of the column. The void volume of a column is typically one third of the total bed volume. The total bed volume of the DE52 column was 15 ml, and therefore an estimate of the void volume is 5 ml. In support of this estimate, no absorbance was measured on the elution profile until approximately 5 ml of eluent, representing unbound

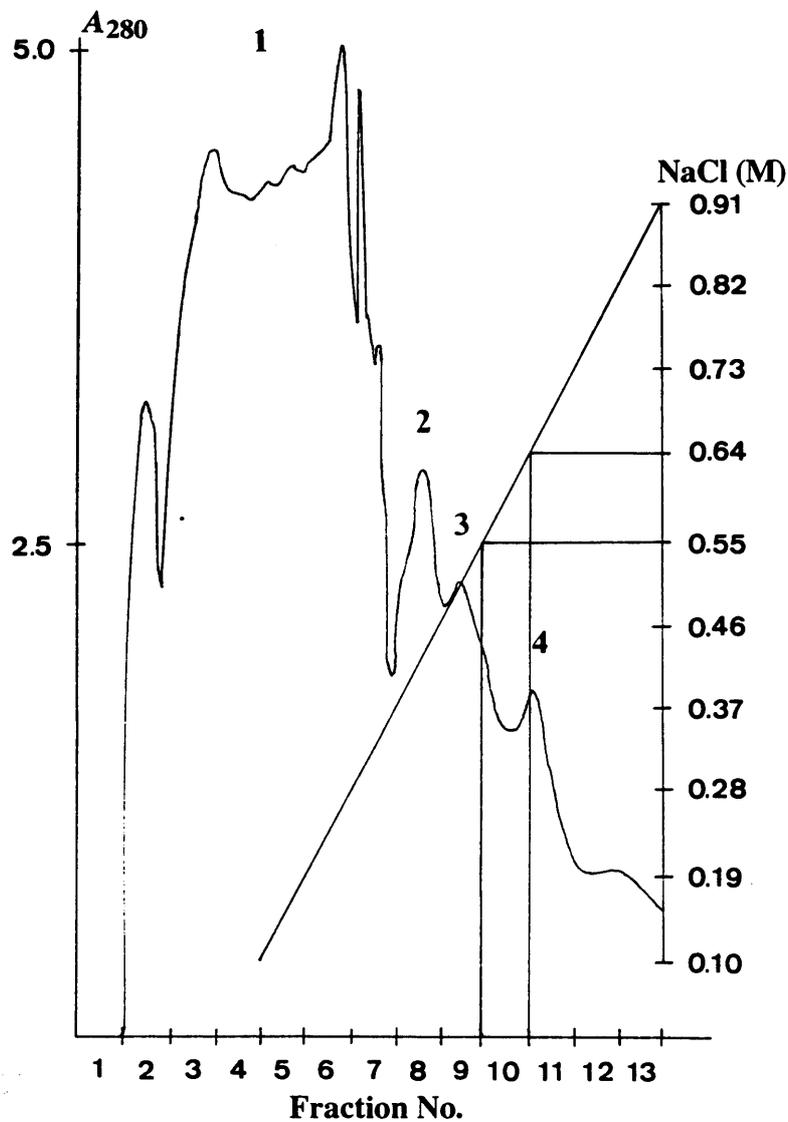


Figure 4.1. Continuous ionic strength gradient elution profile of a *K. lactis* 15 K supernatant on a 15 ml DE52 column.

Preparation of the sample, and column running conditions, were as described in Section 4.2.1. Fractions (5 ml) were monitored by absorbance at 280 nm in a 20 mm flow cell, using a 'Dual Path Monitor UV-2' (Pharmacia) and a 'REC 2' two-channel recorder (Pharmacia). The gradient was from 0.1 M NaCl to 0.91 M NaCl over a 45 ml gradient volume. Four major peaks were observed. K1 and K2 were eluted in peak 4. Refer also to Figure 4.2.

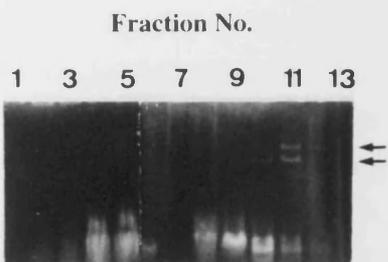


Figure 4.2. Killer plasmid elution profile on application of a continuous ionic strength gradient to a 15 ml DE52 column.

Preparation of the sample, and column running conditions, were as described in Section 4.2.1. 5 μ l of each fraction was analysed by 0.9% agarose/*in situ* lysis gel electrophoresis. Fractions 1 to 4 represent sample loading, and fractions 5 to 13 represent application of the 0.1 M to 0.91 M NaCl gradient. K1 and K2 were visible in fractions 10 to 12, corresponding to peak 4 of the elution profile (Figure 4.1). Arrows indicate position of killer plasmids.

material, had been collected. Taking this into consideration, it was estimated that the killer plasmids actually eluted between 450 and 540 mM NaCl.

4.2.2 Application of a Stepwise Ionic Strength Gradient to a 15 ml DE52 Column

After estimating the NaCl concentration at which K1 and K2 elute from a DE52 column, it was necessary to analyse the separation achieved between the killer plasmids and cytoplasmic proteins on a step gradient. For this step gradient, 350 mM NaCl was chosen as the final wash step, and 800 mM NaCl was chosen for the killer plasmid elution step.

A *K. lactis* cytoplasmic fraction was prepared as described in Section 4.2.1. The 15 K supernatant (approximately 25 ml) was loaded onto a 15 ml, 1 cm diameter, DE52 column, previously equilibrated with 0.1 M NTE, at a flow rate of approximately 0.5 ml min⁻¹. 25 ml 0.1 M NTE was then applied to the column at the the same flow rate, followed by 50 ml 0.35 M NTE (20 mM Tris-HCl, 350 mM NaCl, 1 mM EDTA, pH 7.5), and 50 ml 0.8 M NTE (20 mM Tris-HCl, 800 mM NaCl, 1 mM EDTA, pH 7.5). Running buffers were kept on ice. 5 ml fractions were collected, and samples of each analysed by 10% SDS-PAGE (Figure 4.3).

Figure 4.3 shows the protein elution profile of the DE52 column, as visualised by Coomassie Brilliant Blue R-250 (CBB R-250) stained 10% SDS-PAGE. The majority of the protein either did not bind the DE52 column in the presence of 100 mM NaCl, or was washed off with 350 mM NaCl. 25 µl of each sample was also analysed by 0.9% agarose/*in situ* lysis gel electrophoresis (results not shown). K1 and K2 were eluted with 800 mM NaCl, and were concentrated from 25 ml, down to a maximum of 10 ml. Only a small amount of protein was visible in those fractions containing K1 and K2. From CBB R-250 stained SDS-PAGE (Figure 4.3), a visual estimate that the killer plasmids were effectively separated from 95% of contaminating protein was made.

The above experiment did not reproduce well. Sometimes, K1 and K2 were observed in the flow through. However, the addition of 200 µg ml⁻¹ RNase A to the lysis buffer resolved this problem. Processing a 1 L IFO1267 culture must have overloaded the 15 ml DE52 column. RNA would compete with the killer plasmids for the charged groups of the DE52 resin. The addition of RNase A removed this competition, allowing more of the killer plasmids to bind.

Increasing the 350 mM NaCl wash step to 400 mM NaCl resulted in the elution of approximately 40% (visual estimate from 0.9% agarose/*in situ* lysis gel electrophoresis) of

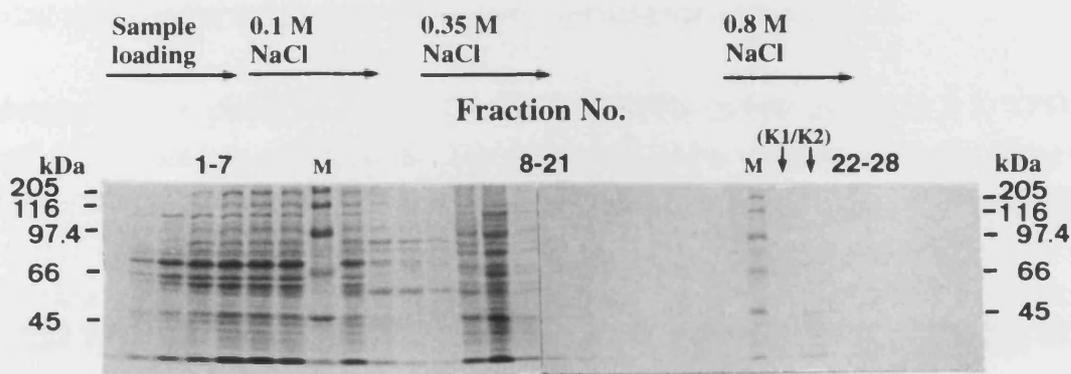


Figure 4.3. Protein elution profile of a *K. lactis* 15 K supernatant on application of a stepwise ionic strength gradient to a 15 ml DE52 column.

Preparation of the sample, and column running conditions, were as described in Section 4.2.2. A total of 28 fractions were collected, and 2.5 μ l of each was analysed by CBB R-250 stained 10% SDS-PAGE. Fractions 1 to 5 represent sample loading, fractions 6 to 10 represent washing with 100 mM NaCl, fractions 11 to 20 represent washing with 350 mM NaCl, and fractions 21 to 28 represent elution with 800 mM NaCl. Molecular weight standards (M) were MW-SDS-200 (Sigma). K1 and K2 were visible by 0.9% agarose *in situ* lysis gel electrophoresis in fractions 22 and 23 (results not shown).

K1 and K2 (results not shown). However, this was not observed when a larger bed volume was used with respect to the size of culture being processed (see Section 4.2.3).

4.2.3 Scale-Up of DE52 Column Chromatography

A bed volume of 120 ml was chosen for the DE52 column for the purposes of scale-up. It was anticipated that a column of this size would be suitable to process *K. lactis* cultures of up to 4 L. In the following experiment, a 2 L *K. lactis* culture was used, and RNase A was added to the lysis buffer. Tris-HCl buffer was replaced by MOPS buffer for its greater pH stability with varying temperature and greater buffering capacity at pH 7.5.

Spheroplasts were prepared from a 2 L *K. lactis* YPD culture grown in 2 L YPD as described in 'Materials and Methods'. The spheroplast pellet was resuspended in 50 ml 0.1 M NME (50 mM MOPS, 100 mM NaCl, 1 mM EDTA, pH 7.5), containing 1 mM PMSF, and 500 μ l 10 mg ml⁻¹ RNase A was added. 5% v/v NP40 was then added drop wise to the spheroplast suspension to a concentration of 0.5%, and stirred on ice for 10 min using a glass rod. Cell lysis was followed by light microscopy, and the lysate centrifuged at 15 K, 4°C, 15 min, in a Sorvall SS-34 rotor. The supernatant (approximately 70 ml) was loaded onto a 120 ml, 5 cm diameter, DE52 column, previously equilibrated with 0.1 M NME, at a flow rate of 2 ml min⁻¹. The column was washed with 250 ml 0.1 M NME at the same flow rate, followed by 450 ml 0.35 M NME (50 mM MOPS, 350 mM NaCl, 1 mM EDTA, pH 7.5), and the killer plasmids were then eluted with 0.8 M NME (50 mM MOPS, 800 mM NaCl, 1 mM EDTA, pH 7.5). All steps were performed between 0 and 4°C. Fractions (12 ml) were monitored by absorbance at 280 nm, and samples of each analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 4.5). Those fractions containing K1 and K2 were pooled. Equivalent sample volumes of the 15 K supernatant and the pooled DE52 fractions were analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 4.6).

Figure 4.5 shows the nucleic acid elution profile as visualised by 0.8% agarose/0.2% SDS gel electrophoresis. K1 and K2 were visible in fractions 71 to 77, but the majority of the killer plasmids were found in fractions 72 to 75, corresponding to peak 3 of the elution profile (Figure 4.4). The killer plasmids, or other nucleic acid, were not detected in any of the other fractions. By comparison of lanes 1 and 2 of Figure 4.6, recovery of K1 and K2 was greater than 90% (visual estimate).

Further experiments were performed to optimise the conditions for DE52 column chromatography. Spheroplast lysis was performed in the presence of 300 mM NaCl, and the 15 K supernatant loaded directly onto a 120 ml DE52 column previously equilibrated with 300 mM NaCl. At this salt concentration, less protein bound to the column, thus

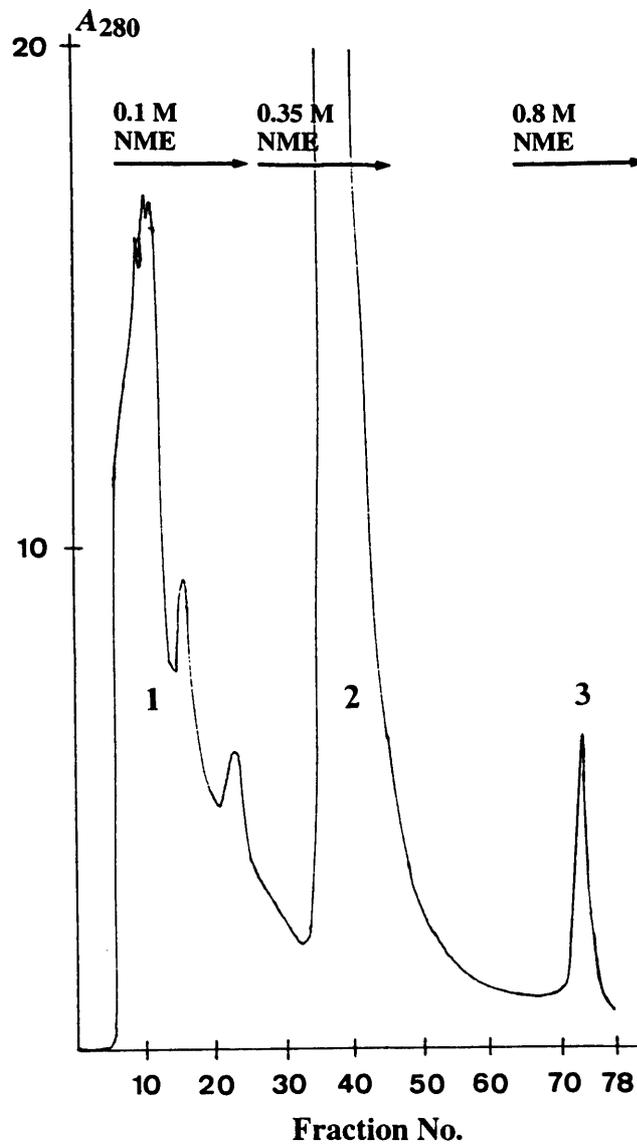


Figure 4.4. Stepwise ionic strength gradient elution profile of a *K. lactis* 15 K supernatant on a 120 ml DE52 column.

Preparation of the sample, and column running conditions, were as described in Section 4.2.3. Fractions (12 ml) were monitored by absorbance at 280 nm in a 20 mm flow cell, using a 'Dual Path Monitor UV-2' (Pharmacia) and a 'REC 2' two-channel recorder (Pharmacia). 78 fractions were collected. Fractions 1 to 6 represent sample loading, fractions 7 to 27 represent washing with 0.1 M NME, fractions 28 to 65 represent washing with 0.35 M NME, and fractions 66 to 78 represent elution with 0.8 M NME. Three major peaks were observed. K1 and K2 were eluted in peak 3. Refer also to Figure 4.5.

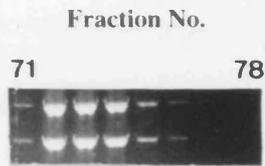


Figure 4.5. Killer plasmid elution profile on a 120 ml DE52 column on application of a stepwise ionic strength gradient.

Preparation of the sample, and column running conditions, were as described in Section 4.2.3. 78 fractions were collected, and 25 μ l of each analysed by 0.8% agarose/0.2% SDS gel electrophoresis (fractions 1 to 70 not shown). K1 and K2 were visible in fractions 71 to 77, corresponding to peak 3 of the elution profile (Figure 4.4).

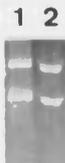


Figure 4.6. Recovery of the killer plasmids after DE52 column chromatography.

Samples were prepared as described in Section 4.2.3. 25 μ l of the 15 K supernatant (approximately 70 ml) (lane 1), and 30 μ l of the pooled DE52 fractions (84 ml) (lane 2), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

increasing the column capacity for the killer plasmids. The column was then washed with 300 mM NaCl, followed by 400 mM NaCl. It was found that washing with 400 mM NaCl did not cause elution of K1 and K2, as was found when a 1 L *K. lactis* culture was processed using a 15 ml DE52 column (see Section 4.2.2). Under these conditions, the 15 ml DE52 column was probably near, or at, capacity.

The majority of K1 and K2 eluted in a volume of 48 ml (fractions 72 to 75 in Figures 4.4 and 4.5), and these fractions were pooled for the second round purification step (see Section 4.3). To sharpen this peak, and thereby improve recovery of the killer plasmids, the NaCl concentration was increased to 1 M, and the flow rate decreased to 1 ml min⁻¹ for the elution step. As anticipated, a 120 ml DE52 column was suitable for processing a 4 L *K. lactis* culture. The final protocol for the DE52 chromatography of the killer plasmids is detailed in 'Appendix I'.

4.3 SECOND ROUND PURIFICATION STEPS

It was estimated that after DE52 column chromatography the killer plasmids were 95% pure (see Section 4.2.2). However, this was not adequate for the purposes of this study, and the killer plasmids needed to be purified further. Contaminating protein could interfere with the analysis of K1 and K2, and in attempts to sequence the TPs.

Protein was visible in those DE52 fractions containing K1 and K2 as visualised by CBB R-250 stained SDS-PAGE (Figure 4.3). This represents a significant amount of protein when compared to the amount of TP present in the sample. Using the calculation in Figure 3.2, it can be estimated that the amount of TP present in the DE52 fractions loaded onto SDS-PAGE (Figure 4.3) was between 1 and 5 ng. CBB R-250 does not detect protein below approximately 0.5 µg protein mm⁻² of gel. The TPs therefore represent a very small percentage of the total protein present in the DE52-purified sample.

A second round purification step was therefore necessary, and four methods were investigated. These were CsCl and sucrose gradient methods, Sephacryl S-1000 column chromatography, and the Qiagen® plasmid purification system. Although CsCl step gradients had already been investigated, it was thought that the DE52-purified killer plasmids, with 95% of contaminating protein removed, might behave differently on these gradients.

DE52-purified killer plasmids were generally pooled into a volume of 48 ml. For some of the following experiments, this volume was too large. A quick and reliable method for the concentration of K1 and K2 was therefore required. Also, in the design of a second round

purification step, the high salt concentration (1 M NaCl) of the killer plasmid sample had to be considered.

4.3.1 Concentration of the DE52-Purified Killer Plasmids

A concentration step is frequently required in protein or nucleic acid isolation procedures to aid purification steps. For example, a smaller volume of solution is easier to handle in precipitation steps or loading onto a chromatography column. Concentration is achieved by removal of water and other small molecules. For the killer plasmids, two methods based on the technique of ultrafiltration were investigated. Ethanol and isopropanol precipitation had initially been attempted, but the resulting pellets did not resuspend well and recoveries were very poor.

Concentration by Ultrafiltration

In ultrafiltration, water and other small molecules are driven through a semi-permeable membrane, which will not allow the large molecules through, by a transmembrane force such as centrifugation or high pressure. Several types of apparatus are available commercially for ultrafiltration on a small scale which use centrifugal force to drive the small molecules through a semi-permeable membrane. One such type of apparatus is the Centriprep™ (Amicon) ultrafiltration device, and this was used in an attempt to concentrate the killer plasmids.

Centriprep concentrators are disposable ultrafiltration devices for samples in the 5 to 15 ml volume range. A filtrate collector is immersed in the sample solution creating a slight hydrostatic pressure which exerts an upward buoyancy force on the membrane at the bottom of the filtrate collector. Centrifugation increases the hydrostatic pressure causing low molecular weight material and solvent to pass through the membrane and into the filtrate collector. Solutes with molecular weights above the membrane cut-off remain within the sample container, becoming more concentrated as centrifugation continues. Ultrafiltration thus occurs in the opposite direction to centrifugal force.

It is common to quote a nominal molecular weight cut-off (NMWC) for an ultrafiltration membrane rather than its pore size. The NMWC is defined as the minimum molecular weight of a globular molecule which will not pass through the membrane. It is important to remember that the shape of the molecule will affect whether it can pass through the pores. Although a globular protein with a molecular weight of 100,000 will not pass through a 100,000 NMWC membrane, a linear molecule such as K2 with a molecular weight of

approximately 9×10^6 may pass through the membrane. The Centriprep concentrator used in the following experiment had a NMWC of 10,000.

48 ml of DE52-purified killer plasmid from a 2 L *K. lactis* YPD culture (see 'Appendix I'), was split equally and added to the sample container of four Centriprep-10 concentrators. Manufacturer's operating instructions were followed, and the assembled concentrator spun at $3000 \times g$, 4°C , in a Jouan CR411 centrifuge until fluid levels inside and outside the filtrate collector had equilibrated. After reaching the equilibration point, the filtrate was decanted and the sample spun again. This procedure was repeated until the sample had been concentrated to the desired volume. To recover the retentate, the Centriprep-10 concentrator was disassembled and the sample removed using a pipette. Equivalent volumes of original sample, filtrate and retentate were analysed by 0.8% agarose/*in situ* lysis gels (results not shown).

Concentration by Dialysis

Although not strictly defined as ultrafiltration, the principles of concentration by dialysis are similar. With this method, the sample solution is placed in a bag of dialysis tubing, which is placed in a solution or powder that draws water through the dialysis membrane by osmosis. Polyethylene glycol (PEG) is frequently used, and was used in the following experiment in an attempt to concentrate the DE52-purified killer plasmids.

48 ml of DE52-purified killer plasmid, from a 2 L *K. lactis* culture, was split into two equal halves and placed in dialysis bags sealed at one end with a plastic clip. Air was removed from the dialysis bags before the remaining end of the bags were sealed. The bags were then placed in a container of dry PEG 20,000. Approximately every 30 minutes, the dialysis bags were removed from the PEG 20,000 and agitated. As the volume of the sample decreased, the samples were pushed to one end of the dialysis bags, and the plastic clip from the other end moved accordingly. This process was repeated until the combined sample volume was approximately 5 ml. All steps were performed between 0 and 4°C . Equivalent volumes of the DE52-purified killer plasmids and the concentrated sample were loaded onto a 0.8% agarose/0.2% SDS gel (Figure 4.7).

Using the Centriprep-10 concentrators, the killer plasmids took more than 3 hours to reach a combined volume of 5 ml. K1 and K2 were visible in the both the filtrate and retentate, and appeared degraded (results not shown). Sometimes K1 and K2 were completely degraded. The reason for this was not understood, but may have been due to the long spin time, and insufficient refrigeration of the Jouan CR411 centrifuge.



Figure 4.7. Recovery of the killer plasmids after concentration by dialysis.

The killer plasmids were concentrated using dialysis against dry PEG 20,000 as described in Section 4.3.1. 25 μ l of the DE52-purified killer plasmids (48 ml) (lane 1), and 2.5 μ l of the concentrated sample (approximately 5 ml) (lane 2), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

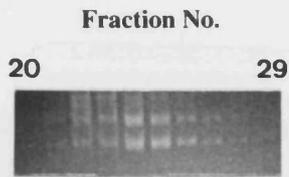


Figure 4.8. Isopycnic centrifugation of DE52-purified killer plasmids on a CsCl-guanidine hydrochloride gradient.

Preparation of the sample, and experimental conditions, were as described in Section 4.3.2. The gradient was fractionated, and 10 μ l of each fraction was analysed by 0.8% agarose/0.2% SDS gel electrophoresis (fractions 1 to 19, and 30 to 34, not shown). K1 and K2 were visible in fractions 21 to 28.



Figure 4.9. Recovery of the killer plasmids from isopycnic gradients.

Samples were prepared as described in Section 4.3.2. 25 μ l of the PEG-concentrated DE52-purified killer plasmids (approximately 10 ml) (lane 1), and 20 μ l of the dialysed CsCl-guanidine hydrochloride gradient-purified killer plasmids (approximately 8 ml) (lane 2), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

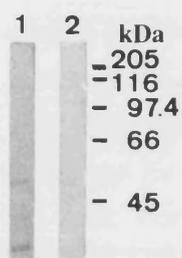


Figure 4.10. Analysis of contaminating protein in killer plasmid samples before and after isopycnic centrifugation.

Samples were prepared as described in Section 4.3.2. 2.5 μ l of the PEG-concentrated DE52-purified killer plasmids (approximately 10 ml) (lane 1), and 2 μ l of the dialysed CsCl-guanidine hydrochloride gradient-purified killer plasmids (approximately 8 ml) (lane 2), were analysed by CBB R-250 stained 10% SDS-PAGE. Molecular weight standards were MW-SDS-200 (Sigma).

experiment, the gradient was run in both the presence and absence of 4 M guanidine hydrochloride.

As described in 'Materials and Methods', DE52-purified killer plasmids were centrifuged using a CsCl starting density of 1.41 g cm^{-3} with, and without, the addition of 4 M guanidine hydrochloride. Those fractions containing K1 and K2 were pooled and dialysed against two changes of 1 L 50 mM MOPS, 1 mM EDTA, pH 7.5, at 1 hourly intervals, at room temperature, and then overnight against 2 L of the same buffer at room temperature. Equivalent sample volumes of the DE52-purified PEG-concentrated killer plasmids and the dialysed CsCl gradient-purified killer plasmids were analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 4.9), and 10% SDS-PAGE (Figure 4.10).

A total of 34 fractions from the CsCl-guanidine hydrochloride gradient were collected. Figure 4.8 shows the banding of K1 and K2 on the CsCl-guanidine hydrochloride gradient. Similar banding of the killer plasmids was observed on the CsCl gradient without guanidine hydrochloride (results not shown). K1 and K2 were visible in fractions 21 to 28, giving a combined volume of approximately 8 ml. The killer plasmids do not appear as sharp bands in Figure 4.8 due to the high concentration of CsCl and guanidine hydrochloride (compare with Figure 4.9, lane 2). Both types of gradient were largely reproducible and, by comparison of lanes 1 and 2 in Figure 4.9, recovery of K1 and K2 was greater than 90% (visual estimate). Contaminating protein was not detected by CBB R-250 stained SDS-PAGE in the dialysed CsCl-purified killer plasmid sample (Figure 4.10).

4.3.3 Centrifugation on Sucrose Gradients

Wilson (1988) has developed a reliable, preparative (500 ml YPD culture of *K. lactis* grown to a density of $5 \times 10^7 \text{ cells ml}^{-1}$), killer plasmid DNA isolation procedure for the purposes of DNA cloning. However, the integrity of the TPs is not maintained with this procedure. The protocol relies on spheroplast lysis using 2% w/v sarkosyl, proteinase K treatment to remove the TPs, and high salt precipitation to remove protein and high molecular weight DNA. By treating with proteinase K before precipitating with salt, it was found that the killer plasmids were better separated from high molecular weight DNA, and their recovery was much improved. At this stage, the killer plasmids could be treated as for normal plasmid DNA, and were phenol extracted to remove remaining protein. After precipitation with ethanol, and resuspension into a suitable volume of buffer, the sample is then loaded onto 10-40% sucrose gradients to further purify the killer plasmid DNA, and to separate K1 from K2. The following procedure attempts to purify killer plasmids with intact TPs on sucrose gradients.

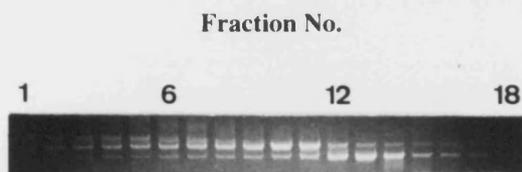


Figure 4.11. Centrifugation of DE52-purified killer plasmids on a 10-40% sucrose gradient.

Preparation of the sample, and experimental conditions, were as described in Section 4.3.3. The gradient was fractionated, and 10 μ l of each fraction was analysed by 0.8% agarose/0.2% SDS gel electrophoresis (fractions 19 to 36 not shown). K1 and K2 were visible in fractions 1 to 18.

As described in 'Materials and Methods', DE52-purified killer plasmids were loaded onto 10-40% sucrose gradients. After centrifugation, fractions of approximately 1 ml were collected and samples of each analysed by 0.8% agarose/0.2% SDS gel electrophoresis (Figure 4.11).

A total of 36 fractions from the sucrose gradient were collected. Figure 4.11 shows the fractionation of K1 and K2 on a 10-40% sucrose gradient as visualised by 0.8% agarose/0.2% SDS gel electrophoresis. The killer plasmids were visible in fractions 1 to 18 of the sucrose gradient, giving a combined volume of approximately 18 ml. Partial separation of K1 and K2 was observed, and the method gave reproducible results. However, for reasons discussed in Section 4.4, no further analysis of this method was performed.

4.3.4 Purification using Qiagen-tips

Qiagen® ('Qiagen Ltd.') is used for the rapid isolation of plasmid DNA from RNA, protein, carbohydrate, and low molecular weight impurities. It is a macroporous anion exchange resin with a hydrophilic surface coating containing DEAE groups, creating an extremely high surface charge density. The large pore size, together with the high density of anion exchange groups, provide a broad separation range which extends from 0.1 to 1.6 M salt. The binding, washing, and elution conditions for Qiagen are strongly influenced by pH. The Qiagen-tip is washed with buffer containing 1 M NaCl at pH 7, and plasmid DNA is eluted with buffer containing 1.25 M NaCl at pH 8.5. At lower pH, a higher salt concentration is required for elution.

At 1 M NaCl, pH 7.5, it was anticipated that the DE52-purified plasmids would bind to the Qiagen resin. The following experiment attempts to utilise the broad separation range of the Qiagen resin to further purify the killer plasmids. Manufacturer's instructions were followed for a Qiagen-tip 500. The Qiagen-tip 500 has a binding capacity of 500 µg of plasmid DNA, which is higher than the expected yield of killer plasmid from a 2 L *K. lactis* culture.

DE52-purified killer plasmids were loaded onto a Qiagen-tip 500 as described in 'Materials and Methods'. After washing and elution, 25 µl of each fraction (4 × 12 ml fractions from loading, 6 × 10 ml fractions from washing, and 1 × 15 ml fraction from elution), were analysed by 0.8% agarose/0.2% SDS electrophoresis. Equivalent volumes of the DE52-purified and Qiagen-purified killer plasmids were analysed by 0.8% agarose/0.2% SDS electrophoresis, and by 10% SDS-PAGE (Figure 4.12).

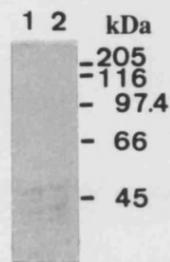


Figure 4.12. Analysis of contaminating protein in killer plasmid samples before and after purification using Qiagen-tips.

Samples were prepared as described in Section 4.3.4. 5 μ l of the DE52-purified killer plasmids (approximately 48 ml) (lane 1), and 1.5 μ l of the Qiagen-purified killer plasmids (15 ml) (lane 2), were analysed by CBB R-250 stained 10% SDS-PAGE. Molecular weight standards were MW-SDS-200 (Sigma).

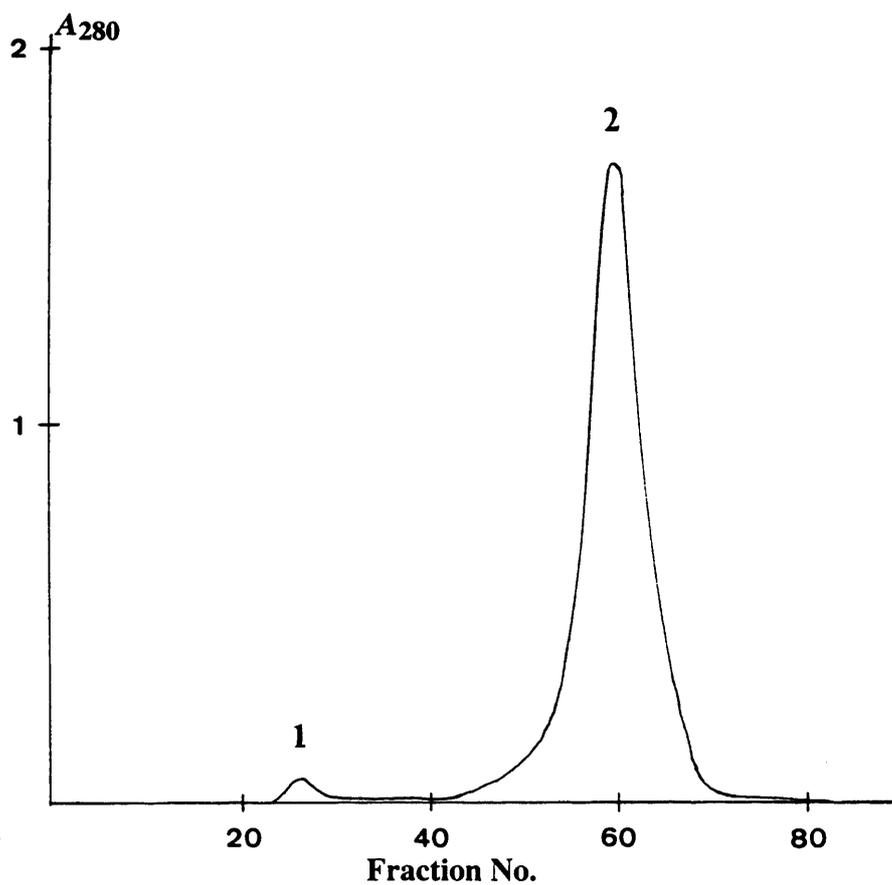


Figure 4.13. Elution profile of DE52-purified killer plasmids on a 320 ml Sephacryl S-1000 column.

Preparation of the sample, and column running conditions, were as described in Section 4.3.5. Fractions (5 ml) were monitored by absorbance at 280 nm in a 20 mm flow cell, using a 'Dual Path Monitor UV-2' (Pharmacia) and a 'REC 2' two-channel recorder (Pharmacia). 88 fractions were collected, and two peaks were observed. K1 and K2 eluted in peak 1. Refer also to Figure 4.14.

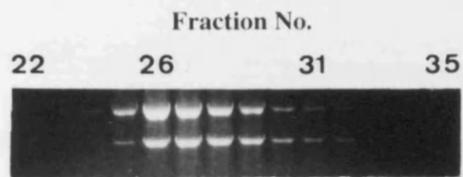


Figure 4.14. Killer plasmid elution profile on a 320 ml Sephacryl S-1000 column.

Preparation of the sample, and column running conditions, were as described in Section 4.3.5. 88 fractions were collected, and 25 μ l of each analysed by 0.8% agarose/0.2% SDS gel electrophoresis (fractions 1 to 21, and 36 to 88, not shown). K1 and K2 were visible in fractions 24 to 34, corresponding to peak 1 of the elution profile (Figure 4.13).

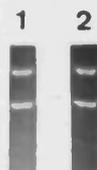


Figure 4.15. Recovery of the killer plasmids after Sephacryl S-1000 chromatography.

Samples were prepared as described in Section 4.3.5. 2.5 μ l of the PEG-concentrated DE52-purified killer plasmids (approximately 5 ml) (lane 1), and 27 μ l of the pooled Sephacryl S-1000 fractions (approximately 55 ml) (lane 2), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

combined Sephacryl S-1000 fractions, and the dialysed Sephacryl S-1000-purified killer plasmids were analysed by 10% SDS-PAGE, and no contaminating protein was detected by CBB R-250 staining in the S-1000 sample (results not shown).

Peaks 1 and 2 of the elution profile (Figure 4.13) were analysed spectrophotometrically. The pool of fractions 24-33 (peak 1) was analysed directly with no dilution. Fractions 45 to 76 (peak 2) were pooled and a tenfold dilution analysed. For both scans, a blank of 1 M NME was used. The scan for elution peak 1 (results not shown, but refer to Figure I.4) gave a profile typical for that of nucleic acid with a peak centred around 258 nm. The scan for peak 2 (results not shown) gave a profile typical for that of protein with a peak centred around 280 nm. The A_{260}/A_{280} ratio of peak 1 was 1.84, a value typical for that of pure nucleic acid.

In chromatography, the degree of separation, or resolution, is defined by:

$$\text{Resolution (R}_s\text{)} = \text{volume between separated peaks} \div \text{average peak volume}$$

Values of R_s greater than 1 indicate baseline separation, but values greater than 1.5 are desirable, especially when one peak is larger than the other. The volumes of peaks 1 and 2 were 50 ml (taken from agarose gel data of Figure 4.14) and 160 ml, respectively, and the separation between the two peaks was 170 ml. Therefore,

$$R_{s(1,2)} = 170 / (50 + 160) / 2 = 1.62$$

This value therefore implies that good baseline resolution between peaks 1 and 2 was achieved.

The procedure was highly reproducible, and was suitable to process a 4 L *K. lactis* culture. However, for this size S-1000 column, the 0.4 M NaCl wash step of the DE52 column was found to be important. If this wash step was not adequate, the protein peak 2 (Figure 4.13) was large, and the resolution between the killer plasmid peak 1 and that of peak 2 was reduced. This is demonstrated in Figure 4.16. Elution profile A was obtained after a long (500 ml) 0.4 M NaCl wash step, and the R_s value was 2.5. Elution profile B was obtained after a short (350 ml) 0.4 M NaCl wash step, and the R_s value was 1.4. Generally, a 450 ml 0.4 M NaCl wash step was adequate.

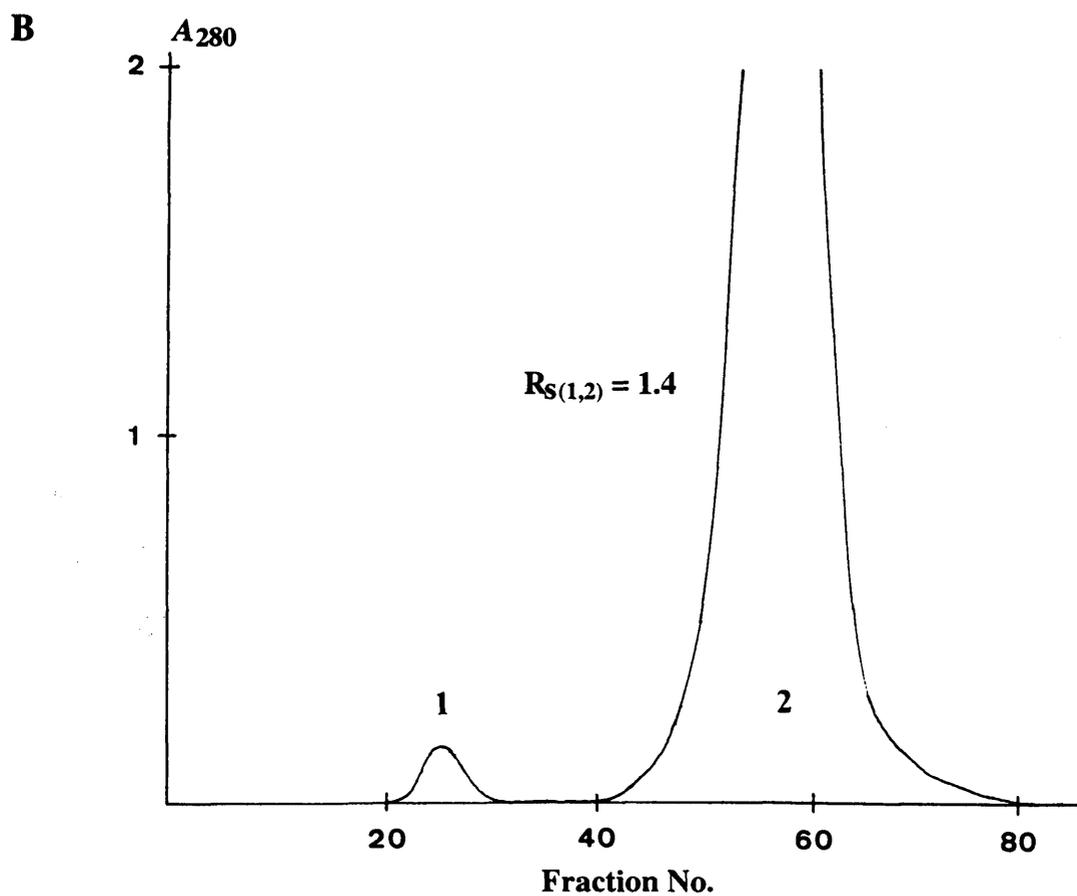
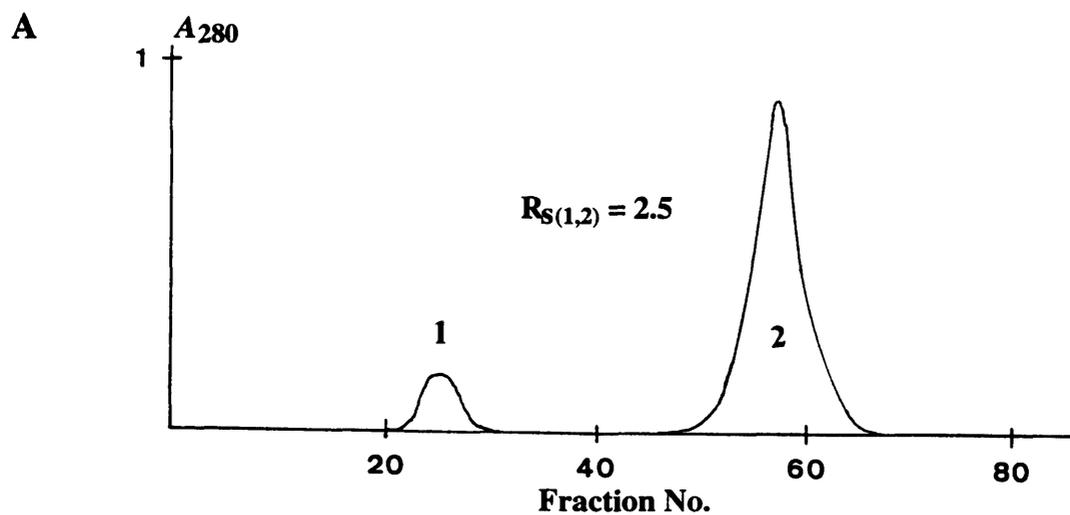


Figure 4.16. Comparison between two DE52 0.4 M NME wash steps, and the effect of resolution on the killer plasmids and contaminating protein separated with Sephacryl S-1000.

Preparation of samples, and column running conditions, were as described in Section 4.3.5. Elution profile A was obtained for the Sephacryl S-1000 column after a 500 ml 0.4 M NME wash step had been applied to the DE52 column, and elution profile B was obtained after a 350 ml 0.4 M NME wash step had been applied.

4.4 CONCLUSION

DE52 column chromatography was successfully developed as a first round procedure for the large scale purification of the *K. lactis* killer plasmids. Following the promising results discussed in Sections 3.4.2 and 3.5, further experiments were performed to examine the behaviour of the killer plasmids and contaminating cytoplasmic protein. It had already been established that the killer plasmids were eluted by 1 M NaCl, but not by 350 mM NaCl (Section 3.4.2). Application of a continuous salt gradient established that K1 and K2 eluted between 450 and 540 mM NaCl. Using these data, a stepwise gradient protocol was developed. Initially, the procedure did not reproduce well as K1 and K2 were sometimes found in the flow through. However, the addition of RNase A to the lysis buffer removed the competition between RNA and the killer plasmids for charged groups of the DE52 resin, allowing more of K1 and K2 to bind. In future preparations, RNase A was routinely added to the lysis buffer to remove this competition. This also eliminated the need to remove RNA at a later stage in the isolation procedure.

Using a 15 ml DE52 column to process a 1 L *K. lactis* culture, a proportion of the killer plasmids eluted with 400 mM NaCl, but not with 350 mM NaCl. However, K1 and K2 were not eluted with 400 mM NaCl when a 120 ml DE52 column was used to process a 2 or 4 L *K. lactis* culture. The smaller column used 15 ml of DE52 resin per litre of original starting culture. The larger column used a minimum of 30 ml of resin per litre of original starting culture. More charged DEAE groups were therefore available for the killer plasmids to bind with the larger column. This probably accounts for why no plasmids eluted (as visualised by 0.8% agarose/0.2% SDS gel electrophoresis) in the 400 mM salt wash when the 120 ml DE52 column was used. Furthermore, MOPS buffer was used in place of Tris-HCl with the 120 ml DE52 column. Its greater buffering capacity at pH 7.5 may have also contributed to the apparently stronger binding of K1 and K2 in the presence of 400 mM NaCl. A further improvement was made in the binding capacity of the DE52 column for K1 and K2 by increasing the starting salt concentration from 100 to 300 mM NaCl. At this higher concentration, less protein bound to the DE52 resin, thereby removing competition with the killer plasmids.

It was estimated from CBB R-250 stained SDS-PAGE that 95% of contaminating protein was removed with 350 mM NaCl, but, as discussed in Section 4.3, a second round purification step was required. However, before a second step could be considered, a reliable concentration method was needed. Firstly, precipitation with ethanol and isopropanol was attempted, but the pellets did not resuspend well and the majority of K1 and K2 was not recovered. Furthermore, for large dilute volumes, ethanol and isopropanol precipitation is not suitable as, with both methods, recoveries are better with a more concentrated sample.

Two other methods for the concentration of the DE52-purified killer plasmids were attempted. The first, using Centiprep concentrators was unsuccessful. Using a 10,000 NMWC membrane, K1 and K2 were found in the filtrate and appeared degraded. However, the use of a membrane with a NMWC smaller than 10,000 was not considered, as even with the membrane used, concentration times were very long (greater than 3 hours). It is likely that the membrane was 'blinded' by solutes packing onto its surface. Also, flow rates across the membrane used are slower at low temperatures. Considerably better results were obtained using the PEG method. Although a 48 ml sample took approximately 3 hours to concentrate down to a volume of 5 ml, recoveries were very good and the killer plasmids appeared intact. Therefore, this method was used, where necessary, to concentrate the DE52-purified sample.

Four methods for the second round purification of the killer plasmids were investigated. Of these, isopycnic centrifugation on CsCl gradients and Sephacryl S-1000 column chromatography gave the best results. The CsCl step gradients, described in Section 3.4.1, were repeated, but no improvement of this method was obtained using the DE52-purified killer plasmids. The Qiagen method gave very good recoveries of K1 and K2, but contaminating protein was not removed. This was not surprising as the method is based on similar principles to that of DE52 chromatography. The broad separation range of Qiagen resin could not be exploited, and protein eluted at the same salt concentration as the killer plasmids.

The sucrose gradient method gave reproducible results, but the separation between K1 and K2 was not as good as that obtained by Wilson (1988). Also, the combined fraction volume (K1 + K2) was approximately 18 ml, compared with approximately 6 ml obtained by Wilson. Significant loss of resolution occurs when a gradient traverses a peristaltic pump, but this cannot alone account for the low resolution between K1 and K2. Moreover, Wilson had also used this method to fractionate the gradient. It is more likely that the gradient was overloaded. Therefore, as a relatively large number of gradients would be required to process a 2 to 4 L *K. lactis* culture, the method was not considered suitable for routine use.

Recovery of the killer plasmids was greater than 90% for both the CsCl isopycnic gradient and Sephacryl S-1000 column. Using both methods, no protein could be detected on CBB R-250 stained SDS-PAGE. However, the reproducibility of the S-1000 column was much greater than the CsCl isopycnic gradient. Also, run times were much faster at approximately 7.5 hours for the killer plasmid containing peak to elute, compared to up to 65 hours for CsCl isopycnic gradient centrifugation. Furthermore, the resolution between the killer plasmid and protein peaks of the S-1000 column was excellent, as long as care had been taken in the 400 mM salt wash of the DE52 column, and the purified sample

gave a typical UV scan for that of pure DNA. For these reasons, Sephacryl S-1000 column chromatography was chosen as the second round purification step for the isolation of K1 and K2.

A new method for the purification of the *K. lactis* linear killer plasmids, based on column chromatography, had thus been developed. Following S-1000 gel filtration chromatography, the plasmids could then be concentrated using the PEG method, and dialysed into an appropriate buffer. The complete method is given in 'Appendix I', and the molecular analysis of purified K1 and K2 is described in 'Chapter 5'.

CHAPTER 5

MOLECULAR ANALYSIS OF THE PURIFIED *KLUYVEROMYCES LACTIS* LINEAR KILLER PLASMIDS

5.1 INTRODUCTION

The large scale killer plasmid purification procedure detailed in 'Appendix I' provided a suitable source of material for the molecular analysis of K1 and K2. Firstly, it was necessary to examine further the integrity of the TPs. Experiments are then discussed that previous purification procedures had not allowed to be performed.

5.2 THE INTEGRITY OF THE K1 AND K2 TERMINAL PROTEINS

During the course of the development of a new purification procedure for the killer plasmids, the integrity of their TPs was analysed by comparison of samples on 0.8% agarose and 0.8% agarose/0.2% SDS, or *in situ* lysis, gels. The presence of TPs appears to inhibit the migration of K1 and K2 into agarose gels. However, if they are first treated with protease, or electrophoresed in the presence of SDS, their migration is normal. Thus, the comparison of samples on 0.8% agarose gels in the presence and absence of SDS could be used as a simple indicator of integrity of the K1 and K2 TPs.

The methods investigated in 'Chapters 3' and '4' were designed to retain the integrity of the TPs, and the killer plasmids recovered did not migrate into normal agarose gels. However, this does not imply that the TPs were fully intact. Some degradation of the TPs may have occurred which was undetectable within the sensitivity range of the previous analysis. The following experiments further examine the integrity of the TPs.

5.2.1 Digestion with Proteinase K

As already discussed, the killer plasmids would not migrate into an agarose gel unless first treated with protease. Throughout 'Chapters 3' and '4', K1 and K2 were electrophoresed in the presence of SDS. The following experiment uses proteinase K digestion as evidence to confirm it is the presence of protein that prevents the migration of the killer plasmids into agarose gels.

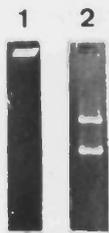


Figure 5.1. Treatment of the purified killer plasmids with proteinase K.

Untreated (lane 1), and proteinase K-treated (lane 2), killer plasmids were analysed by 0.8% agarose gel electrophoresis.

As described in 'Materials and Methods', approximately 2 μ g in total of purified K1 + K2 was incubated with proteinase K. A second sample was incubated under the same conditions without the addition of proteinase K. Both samples were then analysed by 0.8% agarose gel electrophoresis.

As shown in Figure 5.1, the killer plasmids treated with proteinase K (lane 1) were able to migrate into agarose gels. The untreated sample (lane 2) did not enter the gel, and the killer plasmids are clearly visible in the sample well.

5.2.2 Digestion with Endonucleases

Previously published results demonstrated that the killer plasmids could be digested by exonuclease III, but not by λ exonuclease, suggesting that K1 and K2 have TPs covalently attached to their 5' termini (Kikuchi *et al.*, 1984). This experiment was repeated using killer plasmids purified by the new method.

As described in 'Materials and Methods', purified K1 + K2 was incubated with exonuclease III and λ exonuclease. The λ exonuclease digestion also contained DNA of the plasmid pMJR2120, previously digested with *Eco* RI, as a linear DNA control. Samples were withdrawn from the incubation mixture into gel loading buffer containing 0.2% w/v SDS at the times indicated in Figure 5.2, and analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

The killer plasmids were not digested by λ exonuclease, but the linear DNA control was digested (Figure 5.2 A). In contrast, the killer plasmids were digested with exonuclease III (Figure 5.2 B).

5.2.3 Electrophoretic Shift Analysis of TRFs

Terminal restriction fragments (TRFs) of K1 and K2 treated with proteases have been shown to exhibit shifts in their electrophoretic mobilities through polyacrylamide gels (Kikuchi *et al.*, 1984). This observation pointed to the presence of covalently attached TPs. Although it is not possible to estimate the size of the TPs from these results, the differences between electrophoretic mobilities (2 to 12 bp) of protease-treated and untreated TRFs, suggested that the TPs of K1 and K2 were quite small compared to the TPs of other invertrons previously characterised. Furthermore, the experiments suggested that the K1 TP was distinct from the K2 TP. The following experiments were based on this electrophoretic shift analysis. The restriction endonucleases used were *Mae* II, *Msp* I and

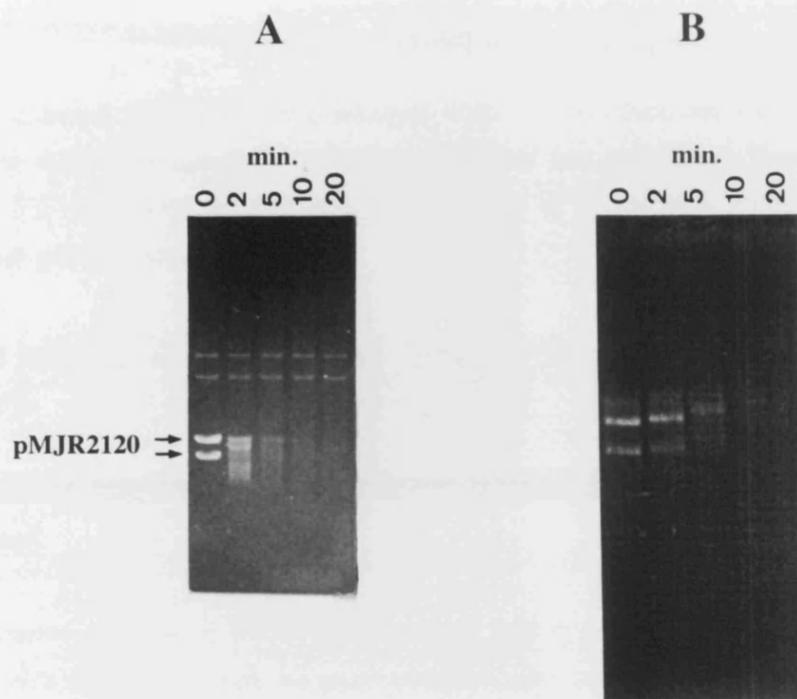


Figure 5.2. Incubation of the purified killer plasmids with endonucleases.

K1 and K2 were incubated with *lambda* exonuclease (A), and exonuclease III (B). Samples were taken at the times indicated, and analysed by 0.8% agarose/0.2% SDS gel electrophoresis. The *lambda* exonuclease digestion also contained the plasmid pMJR2120, previously digested with *Eco* RI, as a linear DNA control.

Xho I. *Mae* II gives TRFs of 53 bp and 92 bp for K1 and K2, respectively. *Msp* I and *Xho* I give TRFs of 139 bp and 121 bp, respectively, for K2. The restriction sites for all the enzymes lie within the TIRs of K1 and K2 (see 'Appendix II'). K1 does not contain cleavage sites for *Msp* I and *Xho* I. The restriction endonucleases were chosen as they leave 3' overhangs which could be filled in, and labelled with ³²P, using the Klenow fragment of *E. coli* DNA polymerase I, and hence visualised by autoradiography.

(i) *Digestion with Xho* I: As described in 'Materials and Methods', K1 + K2 purified from *K. lactis* strain IFO1267 (K1⁺K2⁺), or K2 purified from *K. lactis* strain ABK802 (K1⁻K2⁺), were incubated with *Xho* I, end labelled with ³²P, and then one half of each sample was incubated with proteinase K and the other half left untreated. Each sample was analysed by 5% and 10% PAGE in the presence of 0.2% w/v SDS, or by native polyacrylamide gel electrophoresis.

(ii) *Digestion with Msp* I: The experiment was performed as described in (i) except that *Msp* I was used.

(iii) *Digestion with Mae* II: The experiment was performed as described in (i) except that *Mae* II was used.

In all experiments, untreated TRFs did not enter native polyacrylamide gels. However, DNA bands were observed when the same samples were run in the presence of SDS, with the exception of the K1 and K2 *Mae* II TRFs (see *Gels 3* and *4*), but at a greater molecular weight than would be predicted from their base composition. Proteinase K-treated samples showed different electrophoretic mobilities to untreated samples. The electrophoretic shift was the same irrespective as to whether the samples were run on 5% or 10% PAGE. All preparations of K1 and K2 were analysed by electrophoretic shift analysis to assess the integrity of their TPs. Four gels are described below as examples, and summary, of this analysis. The origin of extra DNA bands arising from the *Mae* II and *Msp* I digests could not be explained (see Section 5.4).

Gel 1 - native polyacrylamide gel electrophoresis (Figure 5.3): The untreated K2 *Xho* I TRF (lane 1) did not migrate into the native polyacrylamide gel. Samples treated with 0.2% (lane 3) or 1% w/v SDS (lane 4) for 10 min at 65°C prior to loading were also unable to migrate into the gel. However, the proteinase K-treated sample (lane 2) gave a K2 *Xho* I TRF migrating at approximately 140 bp.

Gel 2 - Xho I and *Msp* I TRFs (Figure 5.4): A DNA fragment of approximately 280 bp was observed for the K2 *Xho* I TRF (*Xho* I/-) when analysed in the presence of SDS. The proteinase K-treated sample (*Xho* I/PK) gave a DNA fragment of approximately 135 bp.

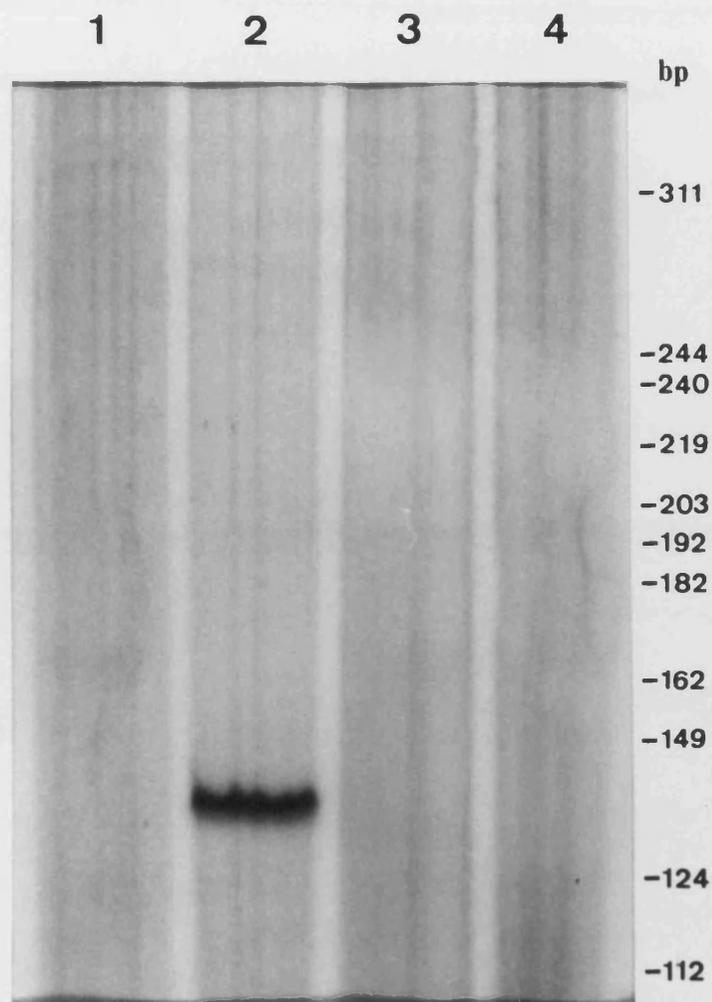


Figure 5.3. Analysis of K2 *Xho* I TRFs by 5% PAGE.

3' end labelled K2 *Xho* I TRFs treated with (lane 2), or without (lane 1), proteinase K, and 0.2% w/v (lane 3), or 1% w/v (lane 4), SDS for 10 min at 65°C, were analysed by 5% PAGE. Marker DNA was *Msp* I cut, 3' end labelled, pBR322.

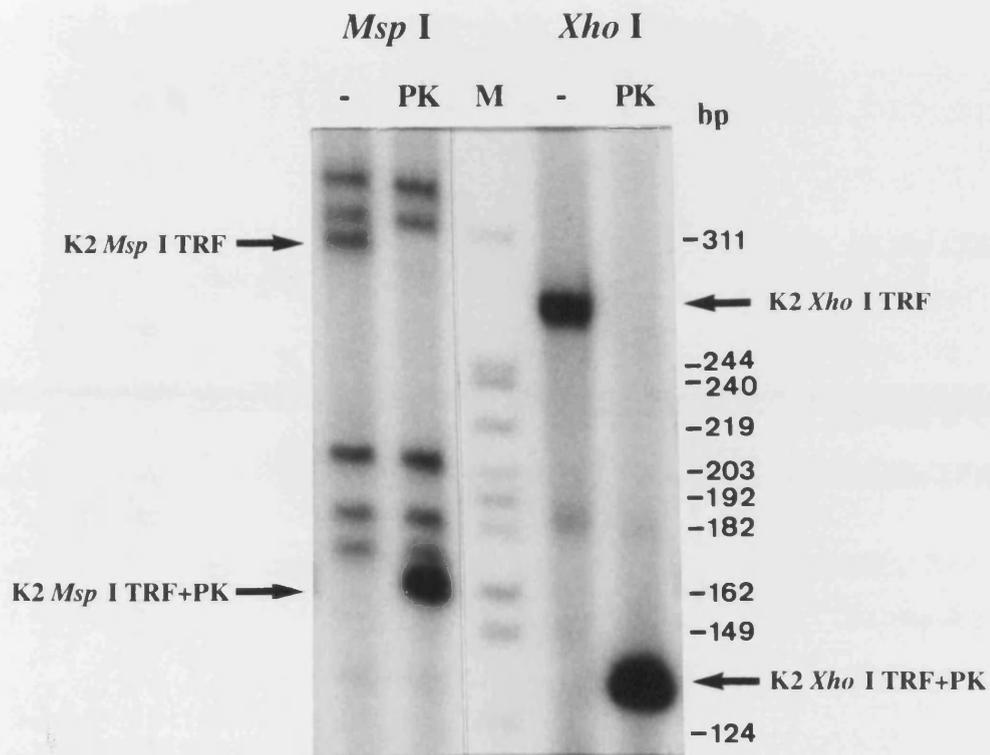


Figure 5.4. Analysis of K2 *Msp I* and *Xho I* TRFS by 5% PAGE in the presence of SDS.

Undigested (-), and proteinase K-digested (PK), 3' end labelled K2 *Msp I* and *Xho I* TRFs were analysed by 5% PAGE in the presence of 0.2% w/v SDS. DNA markers (M) were *Msp I* cut, 3' end labelled, pBR322.

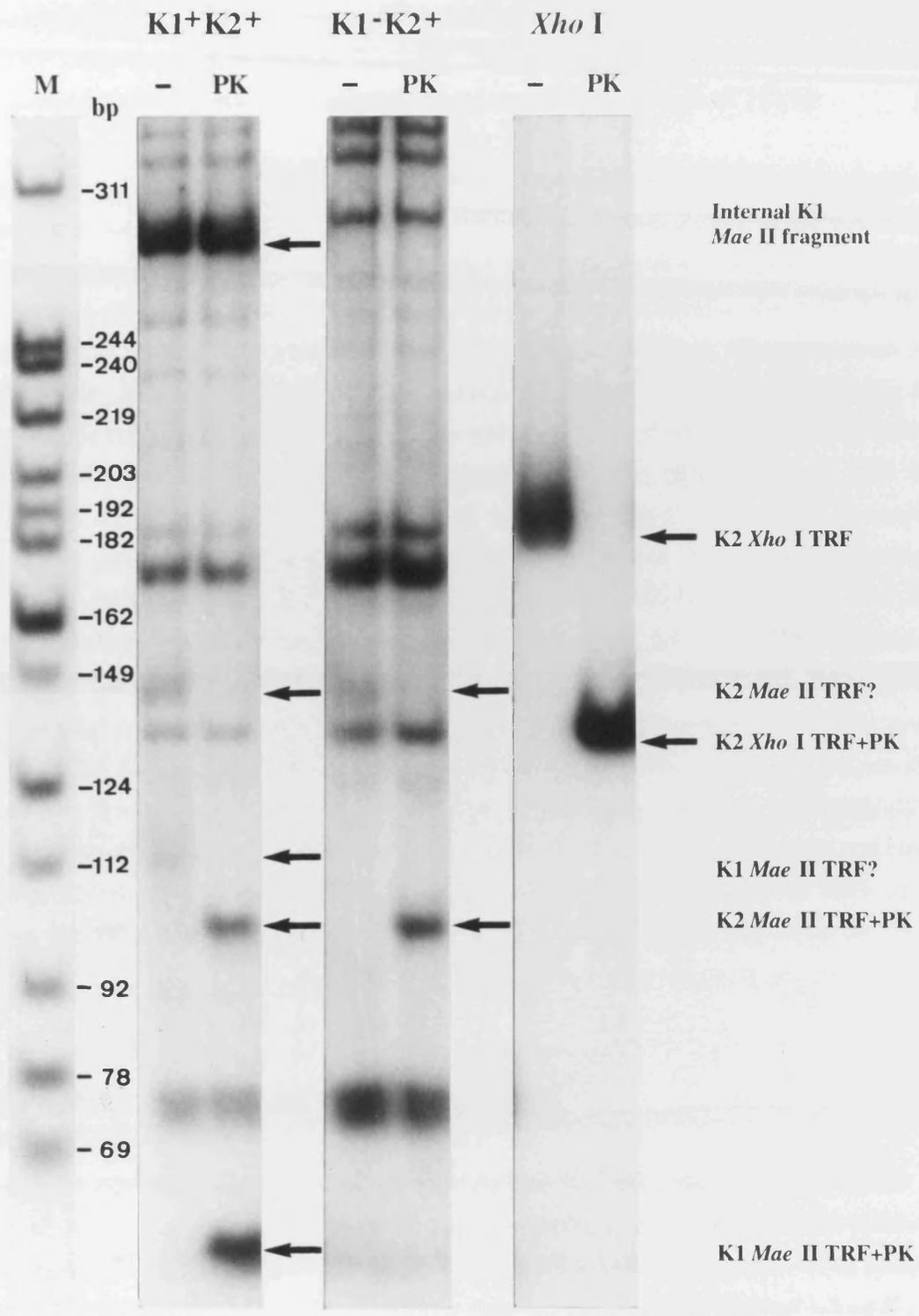


Figure 5.5. Analysis of K1 and K2 *Mae* II TRFs by 5% PAGE in the presence of SDS.

Undigested (-), and proteinase K-digested (PK), 3' end labelled *Mae* II TRFs, derived from killer plasmids purified from *K. lactis* strain IFO1267 (K1+K2+) and ABK802 (K1-K2+), were analysed by 5% PAGE in the presence of 0.2% w/v SDS. Undigested, and proteinase K-digested, *Xho* I TRFs were run as control samples. DNA markers (M) were *Msp* I cut, 3' end labelled, pBR322.

The electrophoretic shift observed for the K2 *Xho* I TRF after treatment with proteinase K was therefore 145 bp. A DNA fragment of approximately 310 bp was observed for the K2 *Msp* I TRF (*Msp* I/-). The proteinase K-treated sample (*Msp* I/PK) gave a DNA fragment of approximately 165 bp, therefore also giving an electrophoretic shift of 145 bp.

Gels 3 and 4 - Mae II TRFs (Figure 5.5; results not shown for *Gel 4*): *Gels 3 and 4* describe the same samples run on 5% and 10% PAGE in the presence of 0.2% w/v SDS, respectively. The K2 *Xho* I TRF (*Xho* I/-) migrated as a duplex DNA of approximately 195 bp, in contrast to a DNA fragment of approximately 280 bp normally observed for this TRF (see *Gel 2*, Figure 5.4) when run on a 5% polyacrylamide gel. The proteinase K-treated sample (*Xho* I/PK) gave a DNA fragment of approximately 140 bp, giving an electrophoretic shift of 55 bp. The same sample run on a 10% gel gave the expected bands and electrophoretic shift. Bands of approximately 55 (K1+K2+/PK) and 100 bp (K1+K2+/PK and K1-K2+/PK) were observed for the K1 and K2 proteinase K-treated *Mae* II TRFs on both the 5% and 10% gels. The DNA fragment of 55 bp was observed with *K. lactis* strain IFO1267 (K1+K2+), but not with strain ABK802 (K1-K2+). The 100 bp DNA fragment was observed with both strains. Therefore, the 55 bp DNA fragment must be attributed to K1, and the 100 bp DNA fragment attributed to K2. Faint DNA fragments migrating at 115 (K1+K2+/-) and 145 bp (K1+K2+/- and K1-K2+/-) were observed for the K1 and K2 *Mae* II untreated samples, respectively, on the 5%, but not the 10% gel. The electrophoretic shift for the K1 *Mae* II TRF, after treatment with proteinase K was therefore 60 bp, and 45 bp for the K2 *Mae* II TRF. This result could not be repeated. Normally, the untreated *Mae* II TRFs were not observed on either 5% or 10% gels. However, the proteinase K-treated TRFs always gave DNA bands as described for these gels.

5.2.4 An Attempt to Visualise the TPs by Silver Staining

Indirect evidence has been presented to demonstrate that proteins are bound to the 5' termini of the killer plasmids (this work and Kikkuchi *et al.*, 1984). Silver staining techniques are claimed to be between 20 and 200 times more sensitive than CBB R-250, and can detect as little as 0.05 to 0.1 ng protein mm⁻² of gel. A total weight of 1.3 µg K1 + K2 will yield approximately 5.5 ng K1 TP and 7 ng K2 TP (refer to Figure 3.2), and should therefore produce a TP yield detectable by this method. Silver staining was used in the following experiment in an attempt to directly visualise the TPs.

As described in 'Materials and Methods', 1.3 µg in total of purified K1 + K2 was digested with DNase I. The digested sample was then analysed alongside an undigested sample, and a sample containing nuclease only, by 10% or 15% SDS-PAGE. Gels were stained with

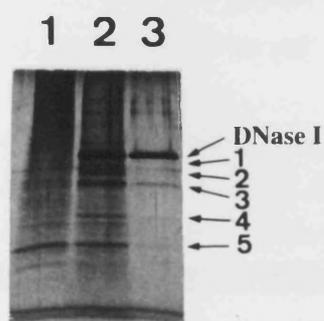


Figure 5.6. Attempted visualisation of the K1 and K2 TPs by silver staining.

1.3 μg in total of purified K1 + K2 was digested with DNase I (lane 2), and analysed by 15% SDS-PAGE. An undigested sample (lane 1), and DNase I sample (lane 3), were run alongside the digested sample. The gel was stained with silver using the National Diagnostics silver staining kit. Numbers refer to protein bands (see Section 5.2.4).

silver, using either the National Diagnostics or Amersham silver staining kits, following the manufacturer's instructions.

TPs were not visualised with any certainty using this method. Figure 5.6 shows the results of a DNase I digestion on 15% SDS-PAGE and using the National Diagnostics silver staining kit for detection. DNase I gave a distinct band corresponding to a molecular weight of approximately 31 kDa. Bands 1 to 4 (refer to Figure 5.6) appeared to be unique to the DNase I-treated killer plasmid sample (lane 2), although faint bands of the same molecular weight as bands 3 and 4 were visible in the DNase I sample (lane 3). Band 4 is probably not of a sufficiently high molecular weight to be either of the TPs. However, any of bands 1, 2 and 3 may represent the K1 TP. If the Stam *et al.* (1986) molecular weight determination of the K2 TP is correct, then this protein would be expected to run above DNase I, but no bands were visible on this part of the gel.

Other gels produced similar results to the gel described above, produced no bands other than that of the nuclease, or produced no extra bands in the DNase I-treated killer plasmid sample. On most gels, a band of approximately 15 kDa (band 5) was visible in both the untreated and DNase I-treated samples (lanes 1 and 2, respectively). Hence, it is possible that an additional, non-covalently associated protein co-purified with the killer plasmids.

5.2.5 Iodination of the TPs

Stam *et al.* (1986) labelled SDS-agarose gel purified K1 and K2 with ¹²⁵I and, after treatment with S1 nuclease, the samples were subjected to SDS-PAGE. Both K1 and K2 appeared each to carry one unique protein, of molecular weights 28 and 36 kDa, respectively. This result demonstrated directly that proteins are covalently bound to the termini of the killer plasmids. Similar iodination experiments are described below to demonstrate that the killer plasmids isolated by the new procedure have intact TPs.

Killer plasmids isolated from a *K. lactis* strain carrying both plasmids (IFO1267), and from a strain carrying K2 only (ABK802), were iodinated using the IODO-BEADS™ Iodination Reagent (Pierce) as described in 'Materials and Methods'. Samples were then either incubated with proteinase K and subjected to SDS-agarose gel electrophoresis, or incubated with nucleases and subjected to SDS-PAGE. The first experiment was designed to demonstrate that protein, and not nucleic acid, is iodinated. The second experiment was designed to directly visualise the TPs. Digestion with a number of nucleases was attempted to determine which one, or combination, gave the best results.

i) *Digestion with proteinase K*: As described in 'Materials and Methods', the iodinated samples were digested with proteinase K. These samples were then analysed alongside undigested samples on a 0.8% agarose/0.2% SDS gel. The gel was stained and photographed as normal, and then dried for autoradiography.

K1 and K2 were visible on the ethidium bromide stained gel in both the proteinase K-digested (PK) and the undigested (-) samples (Figure 5.7 A). Bands corresponding to K1 and K2 were visible on the gel autoradiograph in the undigested (-) samples (Figure 5.7 B). However, no bands corresponding to K1 and K2 were visible in the proteinase K-treated samples (PK). Thus, the proteinase K treatment had removed the ^{125}I label from the DNA, indicating that it was protein, and not DNA, that had been labelled.

ii) *Digestion with nucleases*: As described in 'Materials and Methods', the iodinated samples were incubated with S1 nuclease, mung bean nuclease, micrococcal nuclease, DNase I, and both DNase I and exonuclease III. The samples were then subjected to SDS-PAGE, the gel stained as normal and dried for autoradiography.

A 15 kDa protein was observed in all sample lanes. The protein appeared as an intense band relative to the other protein bands on the gel. The 15 kDa band in the untreated IFO1267 (K1⁺K2⁺) sample (Figure 5.8, lane 1) is faint on this particular gel, but this was not typical. No proteins of the expected molecular weights of the TPs were observed for the untreated samples (Figure 5.8, lanes 1 and 2), or for the samples treated with mung bean nuclease (Figure 5.9, lanes 7 and 8). No distinct bands were observed for the IFO1267 (K1⁺K2⁺) samples treated with DNase I (Figure 5.9, lane 1), or both DNase I and exonuclease III (Figure 5.9, lane 3). However, a distinct band corresponding to a protein of 32 kDa was observed for the ABK802 (K1⁻K2⁺) samples treated under the same conditions (Figure 5.9, lanes 2 and 4 respectively). Distinct proteins of 26.5 and 30.5 kDa were observed for the IFO1267 samples treated with micrococcal nuclease (Figure 5.8, lane 3 and Figure 5.9, lane 5), and with S1 nuclease (Figure 5.8, lane 5). A distinct protein of 30.5 kDa was also observed for the ABK802 sample treated with micrococcal nuclease (Figure 5.8, lane 4 and Figure 5.8, lane 6), and with S1 nuclease (Figure 5.8, lane 6).

The molecular weight of the K1 TP was thus estimated to be 26.5 kDa. The molecular weight of the K2 TP was estimated to be 30.5 or 32 kDa, depending upon the nuclease used to digest the killer plasmid DNA. These results are in contrast to the Stam *et al.* (1986) data.

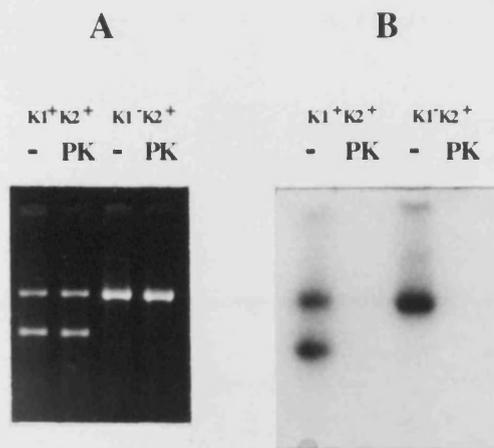


Figure 5.7. Digestion of iodinated killer plasmids with proteinase K.

Undigested (-), and proteinase K-digested (PK), iodinated killer plasmids, purified from *K. lactis* strain IFO1267 ($K1^+K2^+$) and ABK802 ($K1^-K2^+$), were analysed by 0.8% agarose/0.2% SDS gel electrophoresis. A: Ethidium bromide stained gel. B: Autoradiograph.

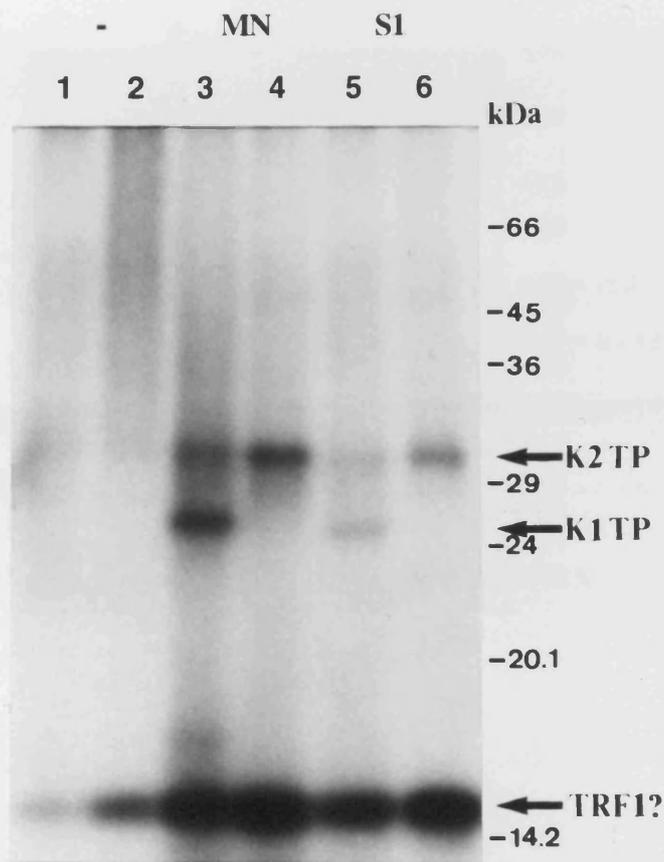


Figure 5.8. Digestion of iodinated killer plasmids with nucleases.

Iodinated killer plasmids purified from *K. lactis* strain IFO1267 (K1⁺K2⁺) (odd numbered lanes) and ABK802 (K1⁺K2⁺) (even numbered lanes), were left untreated (-, lanes 1 and 2), digested with micrococcal nuclease (MN, lanes 3 and 4), or digested with S1 nuclease (S1, lanes 5 and 6), and analysed by 15% SDS-PAGE and autoradiography. Positions of the MW-SDS-70L molecular weight standards (Sigma) were determined from the CBB R-250 stained gel.

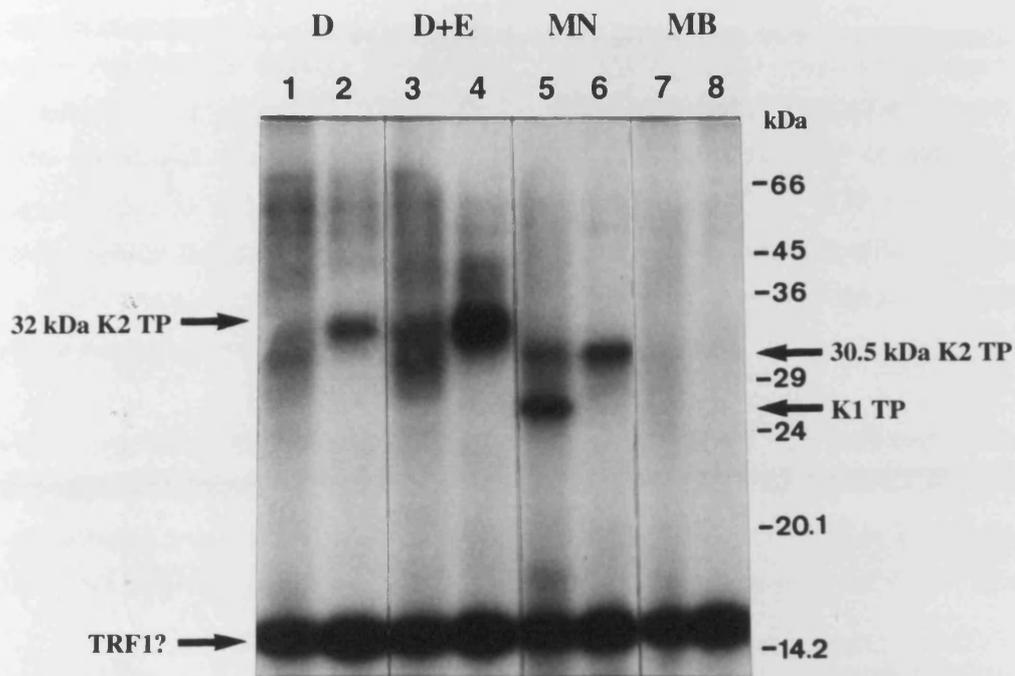


Figure 5.9. Digestion of iodinated killer plasmids with nucleases.

Iodinated killer plasmids purified from *K. lactis* strain IFO1267 (K1⁺K2⁺) (odd numbered lanes) and ABK802 (K1⁻K2⁺) (even numbered lanes), were digested with DNase I (D, lanes 1 and 2), both DNase I and exonuclease III (D+E, lanes 3 and 4), micrococcal nuclease (MN, lanes 5 and 6), or mung bean nuclease (MB, lanes 7 and 8), and analysed by 15% SDS-PAGE. Positions of the MW-SDS-70L molecular weight standards (Sigma) were determined from the CBB R-250 stained gel.

5.3 ANALYSIS OF THE KILLER PLASMIDS

Section 5.2 described experiments to demonstrate the integrity of the TPs. The following experiments attempt to further investigate K1 and K2 and their TPs.

5.3.1 Treatment with β -mercaptoethanol

As already discussed in Section 5.3, although the size of proteins cannot be determined by electrophoretic shift analysis of TRFs, the K2 TRFs appeared to migrate at a much higher apparent molecular weight than would be expected from the size of the K2 TP. β -mercaptoethanol or dithiothreitol are commonly added to SDS-PAGE sample buffer to cleave disulphide bonds which can give rise to anomalous electrophoretic mobilities. K2 *Xho* I TRFs were therefore treated with β -mercaptoethanol, and their electrophoretic mobilities compared with untreated samples.

Untreated and proteinase K-treated ^{32}P labelled K2 *Xho* I TRFs were prepared as described in 'Materials and Methods'. One half of each sample was then treated with 0.1 M β -mercaptoethanol and 1% w/v SDS at 37°C for 3 hr, and the other half left untreated. The samples were then analysed by 5% and 10% PAGE in the presence of 0.2% w/v SDS.

As shown in Figure 5.10, DNA fragments of the expected electrophoretic mobilities were observed for the untreated and proteinase K-treated TRFs (-/- and PK/-, respectively). However, no difference in their mobilities was observed after treatment with β -mercaptoethanol (compare -/- with -/ β , and PK/- with PK/ β) on either 5% or 10% PAGE (results shown are for 5% PAGE).

5.3.2 Treatment with Piperidine

The linkages between the adenovirus and ϕ 29 TPs and their DNA are readily hydrolysed in alkali or by incubation with piperidine (Desiderio and Kelly, 1981; Smart and Stillman, 1982; Hermoso and Salas, 1980; Peñalva and Salas, 1982). This suggested that their DNA-peptide linkages were nucleoside phosphodiester bonds to serine or threonine, but not to tyrosine residues. Similarly, the DHBV DNA-TP linkage was not labile to alkali, suggesting that the linkage is via a phosphodiester tyrosine bond (Bartenschlager and Schaller, 1988). The following experiment used K2 *Xho* I TRFs to investigate the linkage between the K2 TP and its DNA. If the DNA-peptide linkage is via a serine or threonine, then an electrophoretic shift should be observed, after incubation with piperidine, similar to that observed after treatment with proteinase K (see Section 5.2.3).

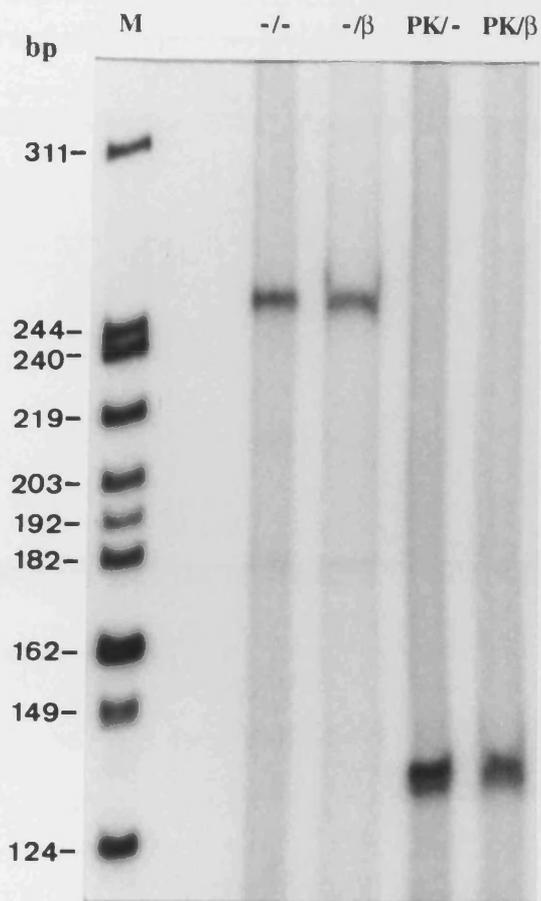


Figure 5.10. Treatment of K2 *Xho* I TRFs with β -mercaptoethanol.

Undigested (-), and proteinase K-digested (PK), 3' end labelled K2 *Xho* I TRFs were left untreated (-), or treated with β -mercaptoethanol (β), and analysed by 5% PAGE in the presence of 0.2% w/v SDS. DNA markers (M) were *Msp* I cut, 3' end labelled, pBR322.

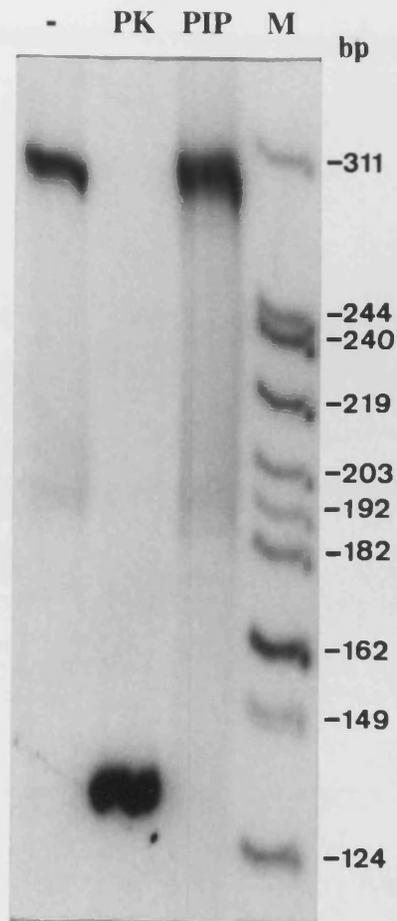


Figure 5.11. Treatment of K2 *Xho* I TRFs with piperidine.

Untreated (-), Proteinase K-treated (PK), and 0.5 M piperidine-treated (PIP), 3' end labelled K2 *Xho* I TRFs were analysed by 5% PAGE in the presence of SDS. DNA markers (M) were *Msp* I cut, 3' end labelled, pBR322.

³²P labelled K2 *Xho* I TRFs were prepared as described in 'Materials and Methods'. One half of the sample was then treated with 0.5 M piperidine at 37°C for 2 hrs, and the other half left untreated. Both samples were then ethanol precipitated and analysed, alongside a proteinase-K-treated sample (see Section 5.2.3), by 5% PAGE in the presence of 0.2% SDS.

As shown in Figure 5.11, the untreated (-), and proteinase K-treated (PK), K2 *Xho* I TRFs gave DNA fragments of the expected electrophoretic mobility. No electrophoretic shift was observed for the piperidine-treated sample (PIP), implying that the K2 TP had not been cleaved from its DNA. The experiment was repeated using 2.5 M piperidine, but still no shift was observed.

5.3.3 Transformation of the Killer Plasmids into the Host Yeast Strain

Killer plasmid gene function has been difficult to study as it has not been possible to isolate K1 and K2, modify their sequence, and transform them back into the host strain. Furthermore, as the expression of killer plasmid genes appears to depend on their own transcriptional system (see Section 1.5.5), cloning of K1 and K2 genes in conventional circular vectors is therefore not suitable for transcriptional studies as such vectors use the host nuclear transcriptional system. *In vivo* homologous recombination has provided a method for the disruption and specific modification of killer plasmid genes (see Section 1.5.6), but an efficient DNA modification and transfer system would fully utilise recombinant DNA technology in research on the biology of the *K. lactis* killer plasmids.

Transfection of *B. subtilis* ϕ 29 DNA (Hirokawa, 1972), adenovirus DNA (Sharp *et al.*, 1976) and *E. coli* Cp-1 DNA (Ronda *et al.*, 1983) into host cells to give viable virus progeny, have all been shown to be sensitive to protease treatment. Moreover, using electroporation, a high frequency transfer system has been developed for PRD1 DNA molecules containing covalently linked TPs into *E. coli* (Lyra *et al.*, 1991). Removal of the TP abolished plaque formation, which could not be rescued by supplying the TP, phage DNA polymerase, or both, *in trans*. It was therefore considered possible to transform killer plasmids which possess intact TPs. If a method could be developed, this would then give rise to the possibility of separating TRFs from internal fragments of K1 and K2 (such a separation should be possible on a Mono Q[®] HR (Pharmacia) column, modifying the internal regions by conventional methods, ligating the modified DNA to the TRFs, and transforming the modified killer plasmids back into the host strain. This would provide a powerful tool for the study of killer plasmid gene function.

Under proper conditions, application of an electric field pulse to cells leads to electroporation, the occurrence of pores or membrane openings through which both ions and molecules can pass. Electroporated cells experience a very rapid (microseconds) membrane discharge (reversible electrical breakdown), followed by a longer lived high permeability state that allows uptake, or release, of molecules (Weaver and Powell, 1989). Electroporation is increasingly used to mediate the uptake of macromolecules into a wide range of cells, including bacteria (Calvin and Hanawalt, 1988), yeast (Delorme, 1989) and mammalian cells (Chu *et al.*, 1987). This has facilitated the transformation of a variety of host cells that could not be transformed by classical methods (Forster and Neumann, 1989), with the advantage that the technique is relatively simple and less time consuming than other DNA transfer methods. Although mainly used to introduce exogenous DNA into cells, electroporation has also been used to cause cellular uptake of protein (Uno *et al.*, 1988). For this reason, it was chosen as the method to attempt the transfer of the killer plasmids into *K. lactis*.

A protocol for the introduction of heterologous DNA into *K. lactis* cells, using a Bio-Rad Gene Pulser, has been described (Bolen and McCutchan, 1990). The conditions used yielded approximately 2000 transformants per μg DNA and, together with the Bio-Rad Gene Pulser User Manual, formed the basis of the experimental procedure described below. The recombinant K1 plasmid rlpSS8 (K1 *ORF2* :: *APT1*) (Soond, 1994) was used in this study. Plasmid rlpSS8 contains the *E. coli* Tn903-derived gene, *APH1*, encoding geneticin[®] (Gibco BRL) (also known as G418) resistance, which can be used as a selectable marker. *APH1* was directed into K1, under control of K1 UCS2, by *in vivo* homologous recombination (see Section 1.5.6), and was stably maintained upon subculturing through non-selective YPD medium after the displacement of native K1 by growth under conditions selective for the recombinant. Plasmid KRp2, an autonomously replicating *K. lactis* plasmid which carries both the *URA3* and *TRP1* genes of *S. cerevisiae* (Wilson, 1988), was used as a transformation control, and *K. lactis* strain SD11 (*Mata*, *trp1*, *lac4*, [K1⁺K2⁺]) was used as the recipient host. A K1⁺K2⁺ strain was used to increase the chances of an introduced recombinant K1 being maintained, as a strain already carrying the killer plasmids would be expected to have the necessary proteins present for replication and transcription.

As described in 'Materials and Methods', *K. lactis* strain SD11 was prepared for electroporation and 100 μl of final suspension used for each transformation. K1 rlpSS8 and K2 in 0.1 M NME were purified from *K. lactis* strain K1-KAN13, and one half of the sample was treated with proteinase K. Aliquots of 0, 5, 50, 100 and 500 ng of K1 rlpSS8 DNA, or the same quantity of control DNA, were used per pulse. DNA and cells were preincubated on ice for 15 min, and then transferred to a 0.2 cm electroporation cuvette. For electroporation, the voltage was 1.5 kV, the capacitance was 25 μF , and the resistance

was 200 Ω . Cold YPD broth was added to the suspension and the cells incubated for 2 hr, 30°C, 300 rpm, before plating. Cells electroporated with killer plasmids were plated onto YPD or YPD containing G418, and cells electroporated with KRp2 were plated onto YPD or SD. All plates were incubated at 30°C for 2 to 4 days.

The pulse duration was 4.4 ms for all of the killer plasmid samples, except for the 500 ng rlpSS8 sample, which dropped to 3.4 ms. The pulse duration for the KRp2 and no DNA samples was 4.8 ms. KRp2 gave a transformation frequency of approximately 2000 transformants per μg DNA when 500 ng DNA was used. This dropped to approximately 1500, 500 and no transformants per μg DNA when 100, 50 and 5 ng DNA, respectively, were used. No transformants were observed for the killer plasmids on YPD plates containing G418. Large numbers of colonies (not counted) were observed for all electroporations plated on YPD medium, indicating that a high percentage of the cells retained viability after the pulse treatment. *K. lactis* cells not incubated with DNA, but subjected to an electric pulse, did not grow on SD media, indicating that the treatment did not induce reversion of the auxotrophic mutations.

5.3.4 An Attempt to Perform Amino-Terminal Sequencing of the K1 and K2 TPs

As mentioned in Section 1.5.4, the genes encoding the TPs have not been identified. However, if the amino acid sequence of the TPs was determined, then their genes could be identified (assuming that they are killer plasmid derived), as both K1 and K2 have been sequenced. A sequence of only five amino acids for each TP would be required.

For the purposes of amino acid sequencing, approximately 0.5 nmol of each TP was required, corresponding to 1.5 and 2.2 mg of K1 and K2 DNA, respectively. Based on the calculation in Figure 3.2, and experimental observations, it was decided to process a 32 L YPD *K. lactis* culture, at a density of 2×10^8 cells ml^{-1} , to provide sufficient material. From results obtained in Section 5.2.5, micrococcal nuclease was the enzyme chosen to digest the killer plasmid DNA and release the TPs. The nuclease produced well defined protein bands for both TPs, digests dsDNA, and has a molecular weight of 18 kDa which is lower than that for both the K1 and K2 TPs. After incubation with nuclease, it was decided to use a Ultrafree-MC Filter Unit (Millipore), with a 5,000 NMWL low-binding, PLCC membrane, to concentrate the sample for loading onto SDS-PAGE and subsequent electroblotting. Ultrafree-MC filters are ultrafiltration devices displaying high recovery rates, and are suitable for the concentration of protein and nucleic acids, and for the removal of solute molecules. If protein bands were detected by staining after blotting, sequencing could then be attempted. It was expected that the 15 kDa protein (see Sections 5.2.5 and 5.4) would be visible, and this would also be sequenced.

A full protocol is given in 'Materials and Methods'. Briefly, eight 4 L *K. lactis* strain IFO1267 cultures were processed as described in 'Appendix I'. Killer plasmid preparations were pooled, ethanol precipitated and resuspended in a final volume of 800 μ l. Micrococcal nuclease was added, and the reaction mixture incubated at 37°C for 3 hr. The sample was then concentrated to 50 μ l using two Ultrafree-MC Filter Units. A sample containing nuclease only, in the same volume and buffer, was also concentrated using this method. Both samples, along with an untreated micrococcal nuclease sample, were then given to J. Kyte, Department of Biochemistry, University of Nottingham, for electrophoresis, blotting and amino-terminal sequencing.

After ethanol precipitation of the pooled preparations, the yield of killer plasmid DNA was approximately 3.5 mg, representing 0.48 nmol of each TP, or 12.5 and 14.5 μ g (calculation uses new molecular weight estimates; see Section 5.4) of the K1 and K2 TPs, respectively. The expected electrophoretic shift was observed after treatment of K2 *Xho* I TRFs with proteinase K, and the killer plasmids would not migrate into an agarose gel unless electrophoresed in the presence of SDS (results not shown). By comparing a retained untreated sample with a sample treated with micrococcal nuclease on a 0.8% agarose/0.2% SDS gel, it was determined that the killer plasmid DNA was fully digested (results not shown). After treatment with nuclease, each sample took approximately 3 hr to concentrate down to approximately 200 μ l using the Ultrafree-MC Filter Units. 200 μ l 0.1 M NME was then added to each sample and concentrated down again to 200 μ l in approximately 3 hr. This process was repeated twice more, taking the same time, and the samples combined after the final wash. The sample was then concentrated to a volume of approximately 40 μ l in approximately 12 hr. The same procedure for the micrococcal nuclease sample took less than 3 hr to concentrate down to the same volume. After blotting, protein bands corresponding to a molecular weight of approximately 18 kDa, were clearly visible, at equal intensities, in the treated and untreated micrococcal nuclease samples. However, the same band was not visible in the killer plasmid plus micrococcal nuclease sample. No bands were visible representing the K1 and K2 TPs, or the 15 kDa protein. Faint bands, which did not give significant sequence data, were visible corresponding to proteins of less than 18 kDa.

5.4 CONCLUSION

In Section 5.2 experiments were described that examined the integrity of the K1 and K2 TPs, and provided new insights into their physical behaviour and the molecular characteristics of K1 and K2. Firstly, the killer plasmids were treated with protease and analysed by agarose gel electrophoresis. Only the sample treated with protease migrated through the gel; the untreated sample remained in the well. This simple test therefore

provided indirect evidence that the TPs were intact. Degraded TPs would not be expected to prevent the entry of the killer plasmids into agarose gels.

It is not known why the presence of TPs prevents the entry of K1 and K2 into agarose gels. Their small size, relative to the size of the DNA to which they are attached, would suggest that charge is not a factor. If killer plasmid molecules became linked, through non-covalent interactions of their TPs, then the plasmids would be too big to enter the agarose gel. The addition of SDS to the buffer would break these interactions, and the killer plasmids would then electrophorese. However, if this hypothesis was correct, it would be likely that single, multimer or circular species would be visible on agarose gels when SDS is not present in the buffer system.

During the development of the new killer plasmid purification procedure it was observed that, under certain conditions, K1 and K2 precipitated (data not shown). For example, precipitation of K1 and K2 sometimes occurred if the killer plasmids were dialysed from a high salt buffer into a buffer containing no salt. Protein precipitates are formed by aggregation of the molecules, and can be induced by a change in pH or ionic strength. If the TPs precipitate, this would then cause precipitation of the TP-DNA complex to occur. It is therefore possible that the killer plasmids were precipitated in the presence of the gel buffer, which has a relatively low ionic strength and higher pH than the buffers used in the purification of the killer plasmids, and the addition of SDS to the gel buffer system maintained the solubility of the TPs.

The terminal structures of the killer plasmids were examined using exonucleases. Exonuclease III, which catalyses the stepwise 3' to 5' removal of 5' mononucleotides from dsDNA carrying a 3'-OH end (Weiss, 1976), was able to digest K1 and K2 progressively. In contrast, λ exonuclease, which catalyses the 5' to 3' stepwise release of 5' mononucleotides from dsDNA with a terminal 5'-phosphate (Little *et al.*, 1967), did not digest the killer plasmids. These results clearly suggested that both K1 and K2 have free 3'-OH ends and blocked 5'-ends, consistent with the presence of 5'-TPs.

Electrophoretic analysis of killer plasmid TRFs gave results in sharp contrast to previously published data, where the TPs were concluded as being quite small compared to the TPs of other invertrons previously characterised. Kikuchi *et al.* (1984) used native polyacrylamide slab gels to analyse the TRFs, whereas this analysis used 0.2% w/v SDS in the gel buffer system. Untreated TRFs did not enter native polyacrylamide gels, even if previously treated with SDS, when killer plasmids purified by the new purification procedure were analysed (see Section 5.2.3). SDS migrates much faster than the TRFs and its removal might cause the TRFs to precipitate (see above). It is possible, therefore, that the killer plasmids used in the Kikuchi *et al.* (1984) study were the products of proteolytic

degradation during preparation of the plasmids, or the gel system used was unable to detect killer plasmids with intact TPs.

Both *Xho* I and *Msp* I TRFs gave an electrophoretic shift of 145 bp for K2 after treatment with proteinase K, compared with a shift of only 8 bp in the Kikuchi *et al.* (1984) study which used a 39 bp K2 *Alu* I TRF. It is unlikely that the differences in these results are due to the TRF used as both *Xho* I and *Msp* I gave rise to the same shift on both 5% and 10% gels. These results thus suggested that K2 purified by the new method had intact TPs.

Digestion of K1 and K2 with *Msp* I and *Mae* II gave rise to bands which could not be explained. These extra bands were identical for each restriction endonuclease irrespective of the strain from which the killer plasmids were purified. This would imply that these bands were not derived from K1, but does not rule out the possibility that they were derived from K2. One possible explanation is that the bands were the products of restriction endonuclease star activity, but this would be expected to occur with K1 as well as K2. It is not possible that the bands were the products of incomplete digestion with proteinase K as they appear in both the untreated and proteinase K-treated lanes. From analysis of the nucleotide sequences of K1 and K2, neither do the bands appear to arise from incomplete restriction endonuclease digestion. Although the most likely explanation would appear to be that the extra bands were the digestion products of another DNA species, no other DNA molecules were detected by agarose gel electrophoresis with the samples used for these experiments. If another DNA species was running at the same molecular weight as the killer plasmids, then this would have been detected on non SDS gels. Some of the extra bands were of the same intensity as the bands derived from the killer plasmids, particularly with the *Msp* I digestions. This would imply that if another DNA species was present, it would be in the same molar ratio as the killer plasmid DNA, or the presence of TPs made the ³²P labelling reaction less efficient for killer plasmid TRFs. However, it is possible that it could be highly repetitious DNA of mobile size and in a small enough quantity to have escaped detection by ethidium bromide staining of agarose gels, but when digested gave a large amount of unit length products. Alternatively, the extra DNA bands may have been an artifact of ³²P 3' end labelling. Restriction analysis of the killer plasmids, and visualisation of the products by 0.8% agarose/0.2% SDS gel electrophoresis, did not give rise to the extra bands (results not shown).

The extra bands arising from digestion with *Mae* II made the analysis of these results particularly difficult. Proteinase K-treated K1 and K2 *Mae* II TRFs gave DNA fragments of 55 and 100 bp, respectively. However, except with the 5% gel analysis described, and even these could not be ascribed with any certainty, untreated *Mae* II TRFs were not detected in any other experiment. On the 5% gel, the K2 *Xho* I TRF gave an electrophoretic shift of 55 bp after treatment with proteinase K, compared with a shift of

165 bp normally associated with this fragment. However, the same sample run on a 10% gel gave the expected shift of 165 bp, implying that the untreated K2 TP was partially degraded during loading and running of the 5% gel. Faint bands were observed for the untreated *Mae* II samples on the 5% gel which were not visible on the 10% gel, giving electrophoretic shifts of 60 and 45 bp for the K1 and K2 *Mae* II TRFs, respectively, after treatment with proteinase K. However, it must be assumed that the TPs of the untreated samples were partially degraded.

Extrapolating data from the *Xho* I and *Msp* I digests, it would be expected that the untreated K1 *Mae* II TRF would give a DNA fragment of approximately 165 bp (estimate from comparison of sizes of K1 and K2 TPs), and the K2 *Mae* II TRF would give a fragment of approximately 245 bp ($100 + 145 = 245$ bp). No DNA fragments of this size were observed for the untreated K1 and K2 *Mae* II TRFs, whereas distinct fragments for the K2 *Xho* I and *Msp* I TRFs were clearly visible. The presence of TPs did not prevent labelling of the *Mae* II TRFs, as proteinase K treatment was performed after the labelling reaction and the treated *Mae* II TRFs were clearly visible. The precipitation of the killer plasmids under certain conditions has already been discussed. However, the addition of SDS prevented this from occurring, and was therefore unlikely to be the reason for the absence of untreated *Mae* II TRFs. Therefore, either the untreated TRFs did not electrophorese, or the TRFs were masked by the presence of the extra bands. From the comparison of intensities of the proteinase K-treated TRFs and extra bands, masking of untreated TRFs appears unlikely.

The *Mae* II TRFs are smaller than the *Xho* I and *Msp* I TRFs. The DNA molecular weights of the 53 bp K1 and 92 bp K2 *Mae* II TRFs are approximately 35 and 60 kDa, respectively. The 30.5 kDa K2 TP (see below) gave an electrophoretic shift of 165 bp. Although the size of proteins cannot be determined by this analysis, this is equivalent to a molecular weight of approximately 110 kDa. The K2 TP therefore appears to greatly influence the electrophoretic mobilities of the untreated *Xho* I and *Msp* I TRFs. At a particular size of DNA, the TPs may therefore prevent the migration of the TRFs into the gel. The majority of proteins bind 1.4 g SDS per 1 g protein (Reynolds and Tanford, 1970), effectively masking the intrinsic charge of the polypeptide chains, so that net charge per unit mass becomes approximately constant. Subsequently, electrophoretic separation by SDS-PAGE is dependent only on the effective molecular radius, which roughly approximates to molecular size. However, some proteins (for example, nucleoproteins) can bind varying amounts of SDS, resulting in anomalous electrophoretic mobilities. Also, although SDS-protein complexes are not titratable between pH 7 and pH 10, the buffer system used in this analysis is different to that normally used in SDS-PAGE, and this can effect protein mobility. Therefore, at a particular size of DNA, TP charge may prevent the entry, or slow migration considerably, of killer plasmid TRFs into polyacrylamide gels.

An attempt was made to detect the TPs by silver staining, but gel bands could not be ascribed with any certainty. However, results obtained with iodinated TPs may explain the failure of TPs to be visualised by this method.

Iodinated K1 and K2 were not visible by autoradiography of analytical agarose gels after protease treatment, but were visible by ethidium bromide staining. This clearly demonstrated that it was the TPs that were iodinated, and not the killer plasmid DNA.

No labelled bands representing the TPs were detectable on SDS-PAGE when iodinated killer plasmids were treated with mung bean nuclease. Also, no distinct bands were obtained for the DNase I, or DNase I and exonuclease III, treated IFO1267 (K1+K2+) samples. However, the same digestions gave a distinct band for the ABK802 (K1-K2+) sample, allowing estimation of the molecular weight of the K2 TP to be 32 kDa. This was in contrast to the results obtained with micrococcal nuclease and S1 nuclease, from which the K2 TP molecular weight was estimated at 30.5 kDa. In contrast to DNase I, both micrococcal nuclease and S1 nuclease gave distinct bands for the K1 TP, and its molecular weight was estimated to be 26.5 kDa.

Mung bean nuclease and S1 nuclease are similar to one another in their physical and catalytic properties (Laskowski, 1980; Vogt, 1973). Both enzymes degrade ssDNA to mono or oligonucleotides with phosphate groups at their 5' ends, and duplex nucleic acids are digested if they are exposed to relatively large amounts of the enzyme. However, there are indications that mung bean nuclease is less severe in its action than S1 nuclease. Therefore, if the killer plasmids were not completely denatured, or had re-annealed, then this would explain why TPs were not detected in the samples incubated with mung bean nuclease.

In the presence of Mg^{2+} , DNase I attacks each strand of DNA independently, and the sites of cleavage are distributed in a statistically random fashion (Melgar and Goldthwaite, 1968). It is therefore possible that different lengths of killer plasmid DNA remained attached to the TPs after digestion with this enzyme, resulting in smearing of these samples. Consistent with this, the high molecular weight background appeared to be greater in those samples treated with DNase I, or both DNase I and exonuclease III, compared with those samples treated with micrococcal nuclease or S1 nuclease. The addition of exonuclease III to the reaction mixture did not reduce the high molecular weight background, but two diffuse bands became visible in the K1+K2+ sample. Thus, it is possible that the DNA tail was protected from exonuclease III by the TPs.

Visualisation of the TPs by iodination had an advantage over silver staining in that the nuclease used, and its impurities, were not detected. DNase I has a molecular weight of

approximately 31 kDa, and could therefore obscure the silver stained 32 kDa K2 TP. Also, the desalting of the iodinated killer plasmid samples using a 'PD-10' column, and the ethanol precipitation step, would have removed any minor impurities. The absolute sensitivity of silver staining has been demonstrated to reveal the presence of minor impurities in nearly all purified protein samples. Together with the high molecular weight background observed for the iodinated killer plasmid samples treated with DNase I, the apparent greater purity of the iodinated samples and the sensitivity of silver staining could explain why distinct bands were not visualised for the TPs on silver stained gels.

Given the uncertainty of the DNase I-derived molecular weight for the K2 TP, it was concluded that the more accurate estimate was obtained from the micrococcal nuclease and S1 nuclease digestions. These molecular weight estimates of 26.5 and 30.5 kDa for the K1 and K2 TPs, respectively, were lower than that obtained by Stam *et al.* (1986). In that study, it was estimated that the K1 and K2 TP molecular weights were 28 and 36 kDa, respectively, after treatment with S1 nuclease. The differences could simply be explained by the molecular weight standards used. However, given the sharper bands observed in this study, compared with the results obtained by Stam *et al.* (1986), the new estimates are likely to be the more accurate.

All iodinated killer plasmid samples gave rise to a 15 kDa protein when subjected to SDS-PAGE, and a protein of the same molecular weight was also detected by silver staining. This protein is not covalently bound to either K1 or K2 DNA as it appeared in the untreated killer plasmid samples. The protein product of K2 ORF10, TRF1, has been demonstrated to bind K1 and K2 DNA (Tommasino, 1991; McNeel and Tamanoi, 1991), and has an apparent molecular mass of 16 kDa (McNeel and Tamanoi, 1991). It is probable that the 15 kDa protein detected by iodination and silver staining is TRF1 which has co-purified with the killer plasmids. If this is correct, then TRF1 is capable of binding K2, or both K1 and K2, in the presence of 1 M NaCl, which was the salt concentration used for Sephacryl S-1000 column chromatography in their purification. Although it was not known how well the individual proteins had labelled with ¹²⁵I or silver stain, from the relative intensity and size of the ¹²⁵I labelled and silver stained 15 kDa proteins, its abundance appeared greater than that of the TPs.

In Section 5.3 two experiments were performed which gave insights into the chemical nature of the K2 TP. The new killer plasmid purification procedure also enabled two further experiments to be performed which had not been previously possible.

Treatment of the K2 *Xho* I TRF with β -mercaptoethanol did not result in an electrophoretic shift. This suggested that no disulphide bonds are present in the K2 TP. Treatment of the same TRF with piperidine also did not produce an electrophoretic shift. This suggested

that the K2 DNA-peptide linkage is a nucleoside phosphodiester bond between the K2 DNA 5' dAMP and the OH of a K2 TP tyrosine residue.

Under the experimental conditions described, the recombinant K1, rlpSS8, could not be transformed back into the host strain. However, the transformation efficiency of KRp2 was comparable to that previously published for pEK2 (Bolen and McCutchan, 1990), and controls gave the expected results.

A more recent study (Sanchez *et al.*, 1993) has analysed the physical and biological parameters involved in the efficient transformation of *K. lactis* by electroporation. This study concluded that the most important parameter was the pulse duration (τ). A sharp peak was obtained around 6.3 ms for the transformation efficiency of KEp6, and variation of 1 ms produced a decrease in the number of transformants of between 70 and 80%. τ cannot be adjusted in an independent way. However, τ changes with resistance of the sample and therefore changes with the size of cuvette, with the volume of the sample, and with the electroporation buffer. Also, the Sanchez *et al.* (1993) study demonstrated that pretreatment of the cells with DTT was important. Experiments carried out with this step omitted yielded only 6% of transformants when compared to standard conditions. Under optimal conditions, between 10^6 and 10^7 transformants per μg KEp6 DNA were obtained, compared with 10^3 transformants per μg pEK2 DNA obtained by Bolen and McCutchan (1990).

It is apparent from these results that the values of τ (3.4 to 4.4 ms for the killer plasmids and 4.8 ms for KRp2) obtained in this study were very low compared to the optimal experimental conditions described by Sanchez *et al.* (1993). Also, pretreatment with DTT would have greatly increased the transformation efficiency. A value for τ of 3.4 ms was obtained with the 500 ng rlpSS8 sample. This sample had a NaCl concentration of 33 mM. Sanchez *et al.* (1993) observed that a change in NaCl concentration from 1 to 25 mM produced a decrease in τ from 6.3 to 4 ms, with a corresponding decrease in transformation efficiency of 90%. The high NaCl concentration in this sample could therefore account for the decrease in τ from 4.4 to 3.4 ms. NaCl and other components of the electroporation buffer could account for the decrease in τ from the value of 4.8 ms observed for KEp2 to that of 4.4 ms observed for the killer plasmids.

Under the experimental conditions described, no conclusions could therefore be drawn regarding the possibility of transforming the killer plasmids back into the host strain. A more detailed analysis is required to examine the effect of pretreatment with DTT and τ on transformation efficiency with the *K. lactis* strain used in this study, and hence the effect of sample volume and electroporation buffer on τ .

As already discussed, the TP buffer appears to have a significant effect on the chemical behaviour of the killer plasmid TPs. Therefore, the choice of electroporation buffer may be critical for the killer plasmids.

At the time the electroporation experiment was performed, recombinant plasmids were only available for K1. However, recombinant K2 plasmids, produced by *in vivo* homologous recombination, are now available (see Section 1.5.6). As K1 relies on K2 for its maintenance, a recombinant K2 may be a better choice for transformation studies.

The yield of killer plasmid DNA, and hence the amount of estimated K1 and K2 TP, after precipitation with ethanol, was regarded as sufficient to attempt amino-terminal sequencing of the killer plasmid TPs. However, no protein was detected after electroblotting. In an electrophoretic shift assay using K2 *Xho* I TRFs, the expected shift in electrophoretic mobility was observed (see Section 5.2.3), and plasmids did not migrate into an agarose gel unless electrophoresed in the presence of SDS, prior to treatment with micrococcal nuclease. Clearly, the protein had degraded during the long spin time in concentrating and washing the sample using the Ultrafree-MC Filter Units. The micrococcal nuclease control sample, which took a much shorter time to concentrate, was not degraded. The Ultrafree-MC Filter Units were not suitable to concentrate the nuclease-treated killer plasmid sample. Presumably, the PLCC membrane was 'blinded' by the high concentration of released nucleotides packing onto its surface. Although loss of protein would be expected, precipitation with acetone may have provided a better method for the concentration of the sample.

Ten amino acid residues of the K2 TP have since been determined by amino-terminal sequencing (Takeda *et al.*, 1996). A similar amino acid sequence was present in the cryptic amino-terminal domain of K2 ORF2, the putative K2 DNA polymerase. This experiment, and its significance, is discussed in 'Chapter 6'.

CHAPTER 6

GENERAL CONCLUSION AND DISCUSSION

There is a consensus amongst researchers who have worked on the *K. lactis* linear killer plasmids that they are notoriously difficult, and frustrating, to study. The presence of protein-linked termini has prevented the development of a method for the *in vitro* modification of their genes and transformation back into the host strain. Also, the killer plasmids appear to encode their own transcription mechanism, and their genes are not correctly expressed by the host transcription system when cloned in conventional circular vectors (see Section 1.5.5). Methods to modify the killer plasmids *in vivo* are now available (see Section 1.5.6), but otherwise work has progressed slowly.

The aim of this work was to purify killer plasmids which could then be used for biochemical, molecular and genetic analysis. In order to fulfill this aim, two factors had to be taken into consideration. Firstly, the purification steps used had to be gentle to retain the TPs in a native state. Secondly, it was necessary to purify much larger quantities of the killer plasmids than described in previously published methods in order to produce sufficient product for the anticipated experiments.

A number of experiments were anticipated. As mentioned in Section 1.6, this study formed part of a wider project to develop an *in vitro* replication system for the killer plasmids. This required the purification of the K1 and K2 DNA polymerases, the isolation of killer plasmids with intact TPs, and the identification of other proteins of the replication system. Killer plasmids with intact TPs in a native state could also be used to attempt transformation of K1 and K2 back into the host strain and, if this were possible, could lead to genetic analysis. Finally, purified killer plasmids could be used to analyse the molecular characteristics of K1 and K2, and their TPs.

The results of these studies are shown in 'Chapter 5'. It is evident from the results that more work is required to determine the feasibility of performing some of the experiments. Also, the proposed development of an *in vitro* replication system could not be attempted as the DNA polymerases could not be expressed and, subsequently, purified (Ambrose, 1993). However, this study has provided new insights into the killer plasmids, and possibilities for future research on K1 and K2.

The overexpression of the K2 DNA polymerase, and fragments of its gene, as fusion or native proteins, was attempted in *E. coli* (Ambrose, 1993). In all cases, protein expression was not detected, except for a resynthesised K2 ORF2 amino-terminal fragment expressed as a GST fusion protein which accounted for over 10% of total cellular protein. However, the same fragment was not expressed as a native peptide. Analysis of *in vitro* transcribed K2 ORF2 revealed the presence of two truncated mRNAs of 1 and 1.4 kb. Furthermore, no protein was detected after expression of a 390 bp amino-terminal fragment, although full-length transcripts were detected *in vitro*. The barrier to expression may therefore have been at both the transcriptional and translational level. Ambrose (1993) suggested that the high A/T content of the killer plasmids may have resulted in fortuitous structures resembling transcriptional initiators and terminators. However, K2 ORFs 1, 5 and 10 have been expressed in *E. coli* (McNeel and Tamanoi, 1991; Tommasino, 1991), and an epitope-tagged allele of K2 ORF5 was overexpressed in the baculovirus system and a 20 kDa protein detected, corresponding in size to the predicted product (Schaffrath and Meacock, 1995). On the basis of the available evidence, it is not possible to conclude whether or not the complete 110 kDa killer plasmid DNA polymerases can be overexpressed for use in the development of an *in vitro* replication system. If they cannot be expressed in *E. coli*, then expression in another system, such as baculovirus, may be possible.

The necessity of keeping the TPs intact, and the apparent biochemical characteristics of the TPs, restricted the use of methods normally used for DNA purification during the development of an isolation procedure for the killer plasmids. However, as with the Stam *et al.* (1986) procedure, the cytoplasmic location of K1 and K2 facilitated their isolation. As well as avoiding the use of harsh ionic detergents, NP40-induced spheroplast lysis retained nuclei intact, and therefore separated the killer plasmids from nuclear DNA. Chromatographic methods then made possible the purification of high quality killer plasmids with intact TPs.

This isolation protocol not only allows new experiments to be performed, but also provides a basis for the purification of other invertrons. It is anticipated that the purification of other invertrons will meet with similar technical difficulties to those encountered for the killer plasmids. The methods employed here will facilitate their purification so that similar experiments can be performed. Furthermore, steps 1 and 2 of the procedure (see 'Appendix I') have already found use in the routine analysis of K1 and K2 where high quality killer plasmid DNA is required (Schaffrath and Meacock, 1996; Schaffrath *et al.*, 1996; Schaffrath and Meacock, 1995; Schaffrath *et al.*, 1995; Soond, 1994).

The experiments used to demonstrate the integrity of the TPs also revealed the potential of performing similar experiments to analyse their molecular properties. In particular, the experiments using killer plasmid TRFs allowed analysis of the linkage between the K2 TP

and its DNA. Furthermore, treatment with different proteases gave rise to TRFs of different electrophoretic mobilities (Kikuchi *et al.*, 1984). Therefore, assuming that the TPs are encoded by the cryptic amino-terminal domains of their respective DNA polymerases (see below), further analysis using specific proteases to map the site around the linking amino acid may provide evidence of which tyrosine residue of the K2 TP forms the phosphodiester bond.

Before further electrophoretic shift analysis of K1 can be attempted, it is necessary to visualise untreated K1 TRFs by PAGE. As suggested in Section 5.4, the size of the TRF may be important, and so untreated K1 TRFs may be visualised if other restriction endonucleases were used to produce them. For example, *Dde* I produces K1 TRFs of 267 and 366 bp. Also, gel buffers are known to effect the migration of protein, and therefore a different buffer system may allow the visualisation of the *Mae* II TRFs.

Analysis with specific proteases, to map the amino acid residue linking the killer plasmid TPs to their DNA, should also be possible with iodinated TPs and would complement a similar study using TRFs. Iodinated TPs could also be used to analyse the linkage between the TPs and their DNA. This would confirm the result obtained with the piperidine-treated K2 *Xho* I TRF, and allow the study of the linkage between the K1 TP and its DNA as the iodinated K1 TP was clearly visible by PAGE.

It was evident from the results obtained (see Sections 5.3.3 and 5.4), and a study analysing the parameters involved in the efficient transformation of *K. lactis* by electroporation (Sanchez *et al.*, 1993), that further analysis would be necessary to determine the feasibility of transforming the killer plasmids back into the host strain by electroporation. It may be possible to transform the killer plasmids by classical methods, such as those using lithium acetate. Since a method for the transformation of K1 and K2 would greatly facilitate the study of the killer plasmids, such experiments should now be attempted.

As mentioned in Section 5.5, the K2 TP has been shown to share an amino-terminal domain of K2 ORF2, the putative plasmid-encoded DNA polymerase (Takeda *et al.*, 1996). The K2 TP was purified from 1 kg wet weight of cells by ammonium sulphate precipitation and two rounds of centrifugation to equilibrium in CsCl gradients. After digestion with DNase I, ten amino acid residues of the TP were sequenced, six of which were present in the K2 ORF2 region, Val₂₂₈ to Trp₂₃₇. However, a number of criticisms must be made about this study.

Takeda *et al.* (1996) estimated that, after two rounds of CsCl isopycnic centrifugation, 9.9 mg of K2 DNA was purified. After digestion with DNase I, 0.8 mg of K2 TP was obtained as determined by the dye-reagent method (Bio-Rad, USA). Although it is reasonable to

assume that 9.9 mg of K2 DNA would be obtained from 1 kg wet weight cells, that amount of K2 DNA would only yield a maximum weight of 80 µg, or 2.2 nmol, of the K2 TP (figure obtained using an average base pair molecular weight of 660 Da, and a molecular weight of 36 kDa for the K2 TP; see also Figure 3.2). Also, the DNase I digestion was performed in a volume of 58 ml, but it is not stated how the sample was concentrated for SDS-PAGE.

As discussed in Section 5.4, the K2 TP appears to run at the same molecular weight as DNase I. However, no controls are presented in the Takeda *et al.* (1996) study. Therefore, their K2 TP sample may not have been homogenous as stated. Furthermore, as discussed in Section 5.4, it is possible that a DNA tail remained attached to the TP after digestion with DNase I, and this may have interfered with the amino-terminal sequencing of the K2 TP.

It was concluded that only six of the ten amino-terminal residues could be unambiguously determined because the K2 TP is fragile and easily degraded after the K2 DNA is removed. However, taking into consideration the above criticisms, this may have been attributable to other factors. Given this, and the fact that the K1 TP has not been sequenced, it would be appropriate to re-attempt the amino acid sequencing of the TPs purified using the method developed in this present study, with the modification of using acetone precipitation to concentrate the TPs as suggested in Section 5.4.

Assuming the Takeda *et al.* (1996) data to be correct, then the location of the K2 TP within the DNA polymerase sequence is surprising. The derived amino-terminal sequence of the K2 TP starts at Val₂₂₈ of K2 ORF2. The highly conserved ExoI region (Blanco *et al.*, 1991) of the K2 DNA polymerase starts at Glu₃₆₀. From the molecular weight of the K2 TP, and taking the average molecular weight of an amino acid to be 110 Da, the ExoI region is therefore located within the TP domain. Furthermore, it is possible that the highly conserved regions ExoII and ExoIII (Blanco *et al.*, 1991), starting at Ile₅₂₅ and Glu₅₄₄, respectively, also overlap with the TP domain. Although the DHBV TP, DNA polymerase and RNase H functions are encoded by the same protein, the TP forms a distinct domain in the amino-terminal quarter of the multifunctional protein (Bartenschalger and Schaller, 1988).

The location of the TP within the DNA polymerase suggests that the K2 TP is derived from a 55 to 60 kDa pTP. The adenovirus 55 kDa TP is derived from a 80 kDa pTP by cleavage late in the infective cycle (see Sections 1.4.1 and 1.4.6). The fate of the adenovirus TP amino-terminal fragment is not clear, but has been shown to interact with the nuclear matrix and postulated to help direct adenovirus replication complexes to the appropriate location within the nucleus (Fredman and Engler, 1993). A killer plasmid pTP

may therefore have a similar function within the cytoplasm or, given the evidence that the killer plasmids can enter the nucleus (see Section 1.5.6), the K2 TP amino-terminal fragment may help direct this. However, such speculations, and their significance to killer plasmid replication, are groundless given the apparent uncertainty of the Takeda *et al.* (1996) data.

The Takeda *et al.* (1996) study also states that the K2 TP was released by treatment with 0.1 M NaOH, but no data was presented to support this. If correct, this would suggest that the K2 DNA-TP linkage is a phosphodiester bond to serine or threonine, in contradiction to results obtained with piperidine in this present study (see Sections 5.4.2 and 5.5). Although the K2 *Xho* I TRF was not treated with alkali, the K2 TP would be released with piperidine under the conditions described if the K2 DNA-TP linkage was via serine or threonine. This, and no supporting data of the Takeda *et al.* (1996) study, would suggest that the K2 DNA-TP linkage is, indeed, via a tyrosine. In support of this, a similar study demonstrated that the DHBV TP is linked to its DNA via a tyrosine residue (Bartenschlager and Schaller, 1988).

Electron microscopic studies of adenovirus and ϕ 29 DNA have identified possible replication intermediates, and shown that their DNAs can form circular and concatemeric molecules by protein-protein interactions of their TPs (see Sections 1.4.2 and 1.4.6). The purified killer plasmids could also be subjected to similar studies. Indeed, initial experiments were performed during this study, and K1 and K2 DNAs were visualised by electron microscopy. However, in the absence of suitable controls, and detailed and sufficient statistical analysis, no data is presented here.

Although invertrons have been found in a wide variety of organisms, little is known about their biology. It is therefore necessary to increase the knowledge of these systems so that comparisons can be made with the best two studied invertrons - adenovirus and bacteriophage ϕ 29. In recent studies, *in vivo* homologous recombination has provided the most powerful tool for the investigation of killer plasmid gene function (see Section 1.5.6). However, a method for the *in vitro* modification of their genes has not been developed, and biochemical analysis of the killer plasmids has proved to be problematic. The experiments presented here attempted to progress these latter two approaches, and the purification of K1 and K2 with intact TPs was an important, and necessary step towards this. The experiments and suggestions described in this study have thus provided a platform from which further progress on killer plasmid biology, and other invertrons, can be made.

APPENDIX I

A NEW METHOD FOR THE LARGE SCALE PURIFICATION OF THE *KLUYVEROMYCES LACTIS* LINEAR KILLER PLASMIDS WITH INTACT TERMINAL PROTEINS

I.1 INTRODUCTION

The procedure for the large scale purification of the *K. lactis* linear killer plasmids with intact TPs was constantly improved over the period of this work. It is presented here in its entirety and final form with an example of a typical preparation (Figures I.1 to I.4, and Tables I.1 to I.3).

I.2 PURIFICATION OF K1 AND K2

Unless otherwise stated, all steps were performed between 0 and 4°C.

Buffers

0.1 M NME: 50 mM MOPS, 100 mM NaCl, 1 mM EDTA, pH 7.5

0.3 M NME: 50 mM MOPS, 300 mM NaCl, 1 mM EDTA, pH 7.5

0.4 M NME: 50 mM MOPS, 400 mM NaCl, 1 mM EDTA, pH 7.5

1 M NME: 50 mM MOPS, 1 M NaCl, 1 mM EDTA, pH 7.5

Step 1: Cell Growth and Preparation of Cytoplasmic Fraction

Cultures of *K. lactis* strain IFO1267 were used as the source of K1 and K2, but other killer plasmid containing strains can be used. Typically, four to eight 500 ml YPD cultures were grown to a density of 2×10^8 cells ml⁻¹ in 2 L Erlenmeyer flasks in an orbital shaker rotating at 275 rpm, at 30°C. The cells were harvested by centrifugation at 4 K, 20°C, 10 min, in a Sorvall GS3 rotor. The pellet was resuspended in 300 ml Q-water per litre of

culture, re-centrifuged, and the wet weight of cells determined. The yeast cells were then spheroplasted to remove their cell walls. A detailed spheroplasting protocol is given in 'Materials and Methods'. The spheroplast pellet(s) was spread evenly around the bottom and along the lower sides of the centrifuge tube, and resuspended in 25 ml 0.3 M NME, containing 1 mM PMSF and 100 $\mu\text{g ml}^{-1}$ RNase A, per litre of original starting culture. A 5% v/v solution of NP40, in the same buffer, was then added drop wise to a final concentration of 0.5%, and the suspension stirred gently for 10 min using a glass rod. Cell lysis was followed by light microscopy. The lysate was then centrifuged at 15 K, 4°C, 15 min, in a Sorvall SS-34 rotor.

Step 2: DE52 Column Chromatography

The 15 K (20,000 \times g) supernatant was loaded onto a 120 ml, 5 cm diameter, DE52 column, previously equilibrated with 0.3 M NME, at a flow rate of 2 ml min^{-1} . The column was washed with 250 ml 0.3 M NME at the the same flow rate, followed by 0.4 M NME until the absorbance began to level towards the baseline (approximately 450 ml). The killer plasmids were eluted with 1 M NME, at a flow rate of 1 ml min^{-1} . 12 ml fractions were collected, and those fractions containing K1 and K2 pooled (approximately 48 ml).

Step 3: Sephacryl S-1000 Column Chromatography

The DE52-purified killer plasmids were split equally in two, and placed in dialysis bags sealed at one end with a plastic clip. Air was removed from the dialysis bags before the remaining end of the bags sealed. The bags were then placed in a container of dry PEG 20,000. Approximately every 30 minutes, the dialysis bags were removed from the PEG 20,000 and agitated. As the volume of the samples decreased, the samples were pushed to one end of the dialysis bags, and the plastic clip from from the other end moved accordingly. This process was repeated until the combined sample volume was approximately 5 ml (about 3 hr). The sample was then loaded onto a 320 ml, 2.6 cm diameter, Sephacryl S-1000 column, previously equilibrated with 1 M NME, at a flow rate of 0.4 ml min^{-1} . The column running buffer used was 1 M NME. 5 ml fractions were collected, and those fractions containing the killer plasmids combined (approximately 50 ml). The purified killer plasmids were then concentrated to the desired volume using the PEG method, and dialysed into an appropriate buffer.

The above purification procedure routinely yielded approximately 100 to 120 μg of killer plasmid per litre of original starting culture, as determined by absorbance at 260 nm (Figure I.4). Using the calculation in Figure 3.2, the theoretical yield was approximately 250 μg of killer plasmid per litre of original starting culture, assuming 50 copies of K1 and K2 per haploid cell. For a yield of 120 μg , the recovery of K1 and K2 is therefore:

$$(120/250) \times 100 \approx 50\%$$

Similarly, a yield of 100 μg gives a value of 40% for the recovery of the killer plasmids, assuming 50 copies of K1 and K2 per haploid cell. The values were consistent with visual estimates from agarose gels (Figure 5.4). If a copy number of 100 is assumed for the calculation, then the recovery of the killer plasmids is approximately 20 to 25%. The calculation assumes that 100% recovery of K1 and K2 is obtained from the 20,000 \times g spin after NP40-induced lysis. From Figure 3.7, this is essentially achieved as the killer plasmids are barely visible in the 20,000 \times g pellet. The estimate of 50 to 100 copies of K1 and K2 per haploid cell (Gunge *et al.*, 1982) is therefore likely to be closer to 50 than 100. The same conclusion can be reached by taking an estimate of the percent recovery of the killer plasmids and the actual recovery from absorbance at 260 nm, and working backwards through the calculation given in Figure 3.2.

I.3 CONCLUSION

A reliable, and highly reproducible, procedure for the large scale purification of the *K. lactis* linear killer plasmids has been developed which routinely yields between 100 and 120 μg of K1 and K2 per litre of original starting culture at 2×10^8 cells ml^{-1} . The procedure is an enormous improvement on previously published methods. As the various steps in the procedure are gentle, the killer plasmids prepared by this method should be suitable for studies requiring non-denatured TPs. However, if required, harsher conditions could be used in the S-1000 chromatography step.

Table I.1. Purification of the killer plasmids by NP40-induced lysis and column chromatography.

	Sample ^a	Yield of killer plasmid DNA	Purity
2 L <i>K. lactis</i> YPD culture at 2×10^8 cells ml ⁻¹			
↓			
Harvest and spheroplast			
↓			
NP40-induced lysis			
↓			
20,000 × g clearing spin			
↓			
Decant supernatant	1 25 µl	~95%	
↓			
DE52 column chromatography			
↓			
Elute with 1 M NaCl	2 24 µl	~80%	~95%
↓			
Concentrate by dialysis	3 2.5 µl	~70%	
↓			
Sephacryl S-1000 chromatography			
↓			
Collect plasmid-containing fractions	4 25 µl	~60%	~100%
↓			
Concentrate by dialysis	5 1.7 µl	~50%	
↓			
Dialyse into 0.1 M NME	6 1.7 µl	~45%	

^a See Figure I.3.

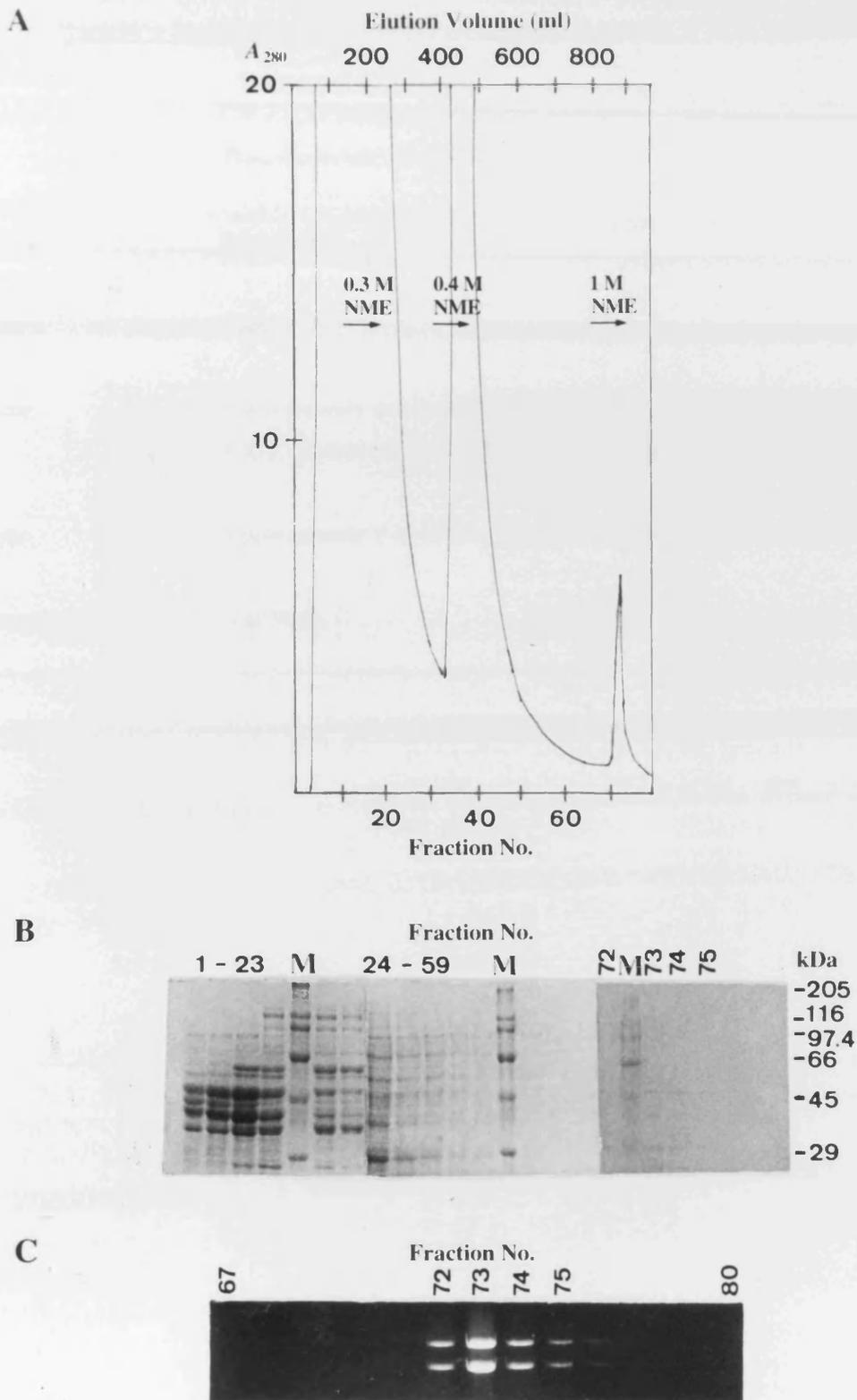


Figure I.1. Purification of the *K. lactis* killer plasmids by DE52 column chromatography.

A. Elution profile (see Table I.2).

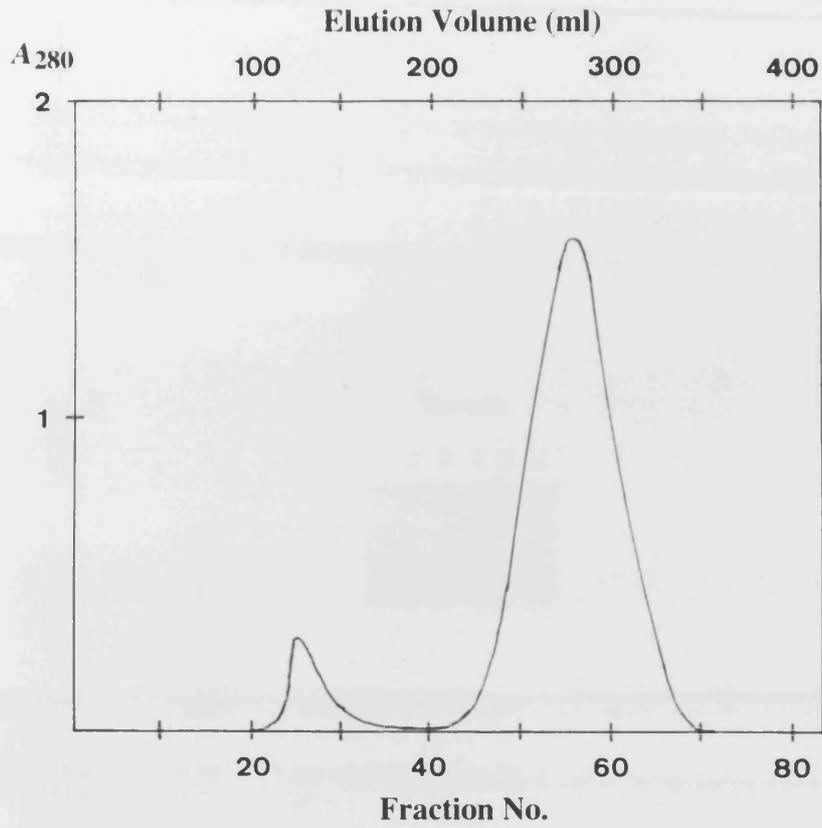
B. 2.5 μ l of selected fractions were analysed by CBB R-250 stained 10% SDS-PAGE. Molecular weight standards (M) were MW-SDS-200 (Sigma).

C. 25 μ l of fractions 67 to 80 were analysed 0.8% agarose/0.2% SDS gel electrophoresis.

Table I.3. Preparative Sephacryl S-1000 Superfine column chromatography of DE52-purified K1 and K2.

Column:	320 ml (2.6 cm internal diameter) Sephacryl S-1000 Superfine (Pharmacia). The column was run at 4°C.
Flow rate:	0.4 ml min ⁻¹ .
Fractions:	5 ml.
Monitor:	Fractions were monitored by absorbance at 280 nm using a 'Dual Path Monitor UV-2' (Pharmacia), and a 'REC 2' two-channel recorder (Pharmacia).
Sample:	Approximately 5 ml of DE52-purified and PEG-concentrated killer plasmids.
Running buffer:	1 M NME.

A



B

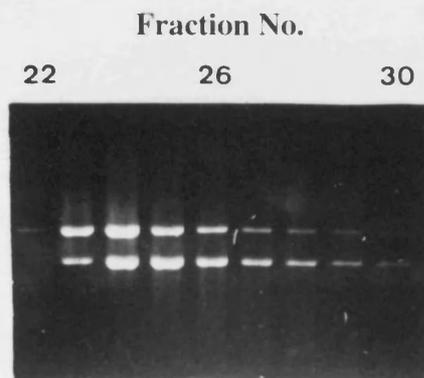


Figure I.2. Purification of the *K. lactis* killer plasmids by Sephacryl S-1000 column chromatography.

A. Elution profile (see Table I.3).

B. 25 μ l of fractions 22 to 30 were analysed by 0.8% agarose/0.2% SDS gel electrophoresis.

Sample

1 2 3 4 5 6

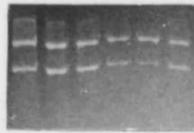


Figure I.3. 0.8% agarose/0.2% SDS gel electrophoresis of *K. lactis* killer plasmids obtained by NP40-induced lysis and column chromatography.

The samples are those described in Table I.1.

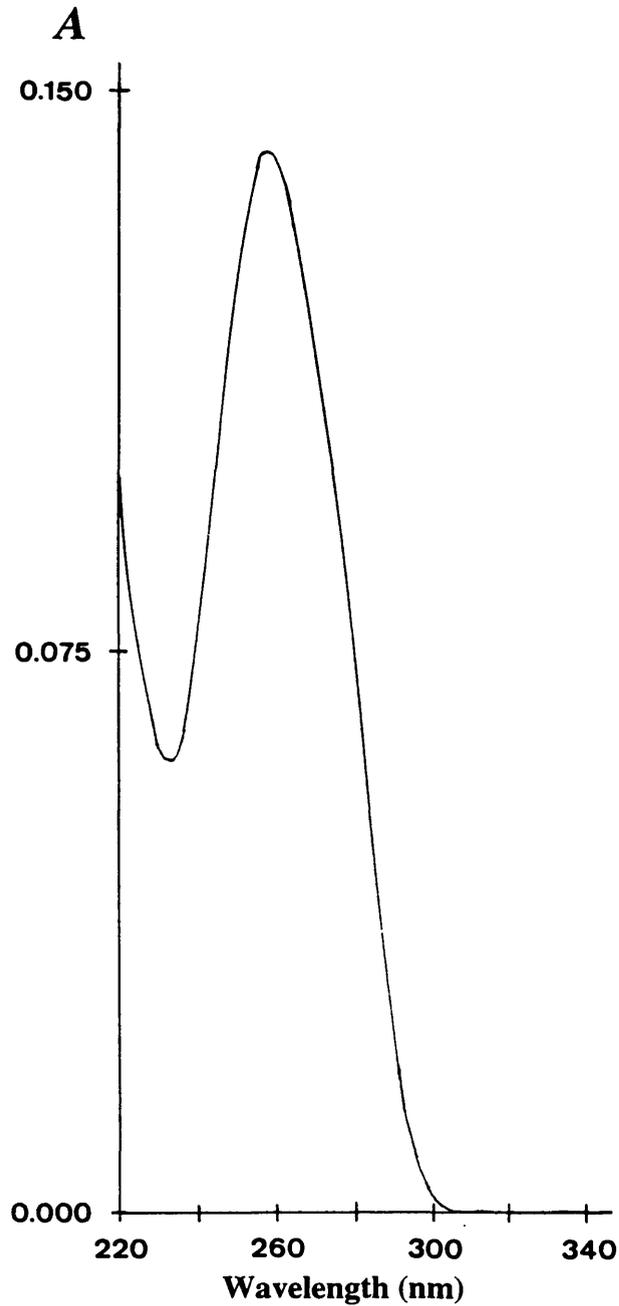
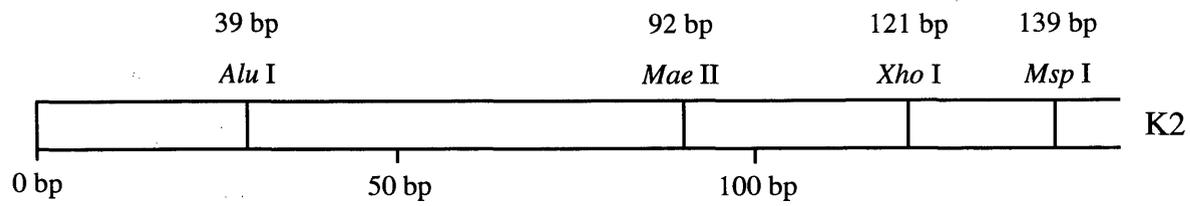
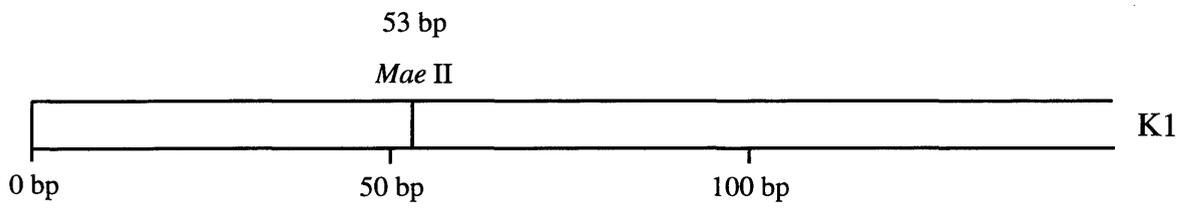


Figure I.4. UV absorption spectrum of killer plasmids purified by the new method.

A tenfold dilution of sample 6 (see Table I.1 and Figure I.3) in 0.1 M NME was analysed using a dual beam Shimadzu Graphicord spectrophotometer. The sample had an absorbance of 0.141 at 260 nm. Using the estimate that an absorbance of 1 corresponds to $50 \mu\text{g ml}^{-1}$ at 260 nm, and given that the volume of sample 6 was 3.3 ml, then approximately 230 μg of killer plasmid was purified from 2 L of original starting culture. The A_{260}/A_{280} ratio was 1.84.

APPENDIX II

PARTIAL RESTRICTION MAPS OF K1 AND K2 TERMINAL INVERTED REPEAT SEQUENCES



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