PLASMIDS FROM *THERMUS* SPECIES

by

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C.L. Koh Plasmids From Thermus Species

Abstract

Currently, in recombinant DNA technology, bacterial plasmids are widely used as the cloning vectors in *Escherichia coli*. An alternative host-vector cloning system based on the extreme thermophilic bacteria would be useful for genetic manipulation experiments.

In order to facilitate the use of plasmid vectors for the construction of recombinant DNA molecules in extreme thermophiles, methods were developed

i) to screen for the presence of supercoiled covalently closed circular plasmid molecules in a number of *Thermus* strains ;

& ii) to isolate them in large quantities.

Plasmids were detected in three of the *T. aquaticus* strains (B1, B2, and H). Their presence in these strains could not be correlated with drug-resistance or bacteriocin production. One plasmid (pCLK1) was isolated from strain B1 and two (pCLK2 and pCLK3) from strain B2. These cryptic plasmids were characterized with restriction endonucleases and their respective physical endonuclease cleavage maps constructed. Plasmid pCLK1 (M.W. 8.76 kb) has single sites for *Bgl*II, *Pst*I, *Sal*I and *Xor*II ; pCLK2 (M.W. 17.24 kb) has single sites for *Hind*III, *Pst*I and *Xba*I ; and pCLK3 (M.W. 9.95 kb) has a single site for *Pst*I.

A hybrid plasmid, constructed between pCLK1 and pHV33, was stably maintained in *E. coli*. A novel method, using the *Staphylococcus aureus/Bacillus subtilis* plasmid pC194, was employed to show that pCLK3 is active in *E. coli*, and to label its cryptic replication region. Attempts were made to clone the leucine genes from *T. aquaticus* H, a strain found to be prototrophic, in *E. coli* but were unsuccessful.

This work has laid the foundation for the development of a hybrid replicon active in *Thermus*, and indicate that a host-vector cloning system in *T. aquaticus* is an attainable goal.

Preface

I thank Prof. Brian S. Hartley, my supervisor, for his continual interest and encouragement during the course of this work.

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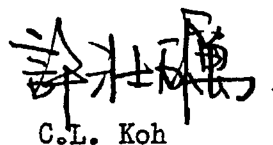
This dissertation and all the work described therein are all my own work.



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Abbreviations Used

Most of the abbreviations used in this dissertation are standard; however, attention is drawn to the following :

A	Adenine (in DNA base sequence)
Ap^R	Ampicillin resistance
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
C	Cytosine (in DNA base sequence)
ccc	Covalently closed circular
Cm^R	Chloramphenicol resistance
CsCl	Cesium chloride
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium bromide
G	Guanine (in DNA base sequence)
GC	Deoxyguanosine and deoxycytosine
HCl	Hydrochloride
kb	Kilobase-pairs
Km^R	Kanamycin resistance
M.W.	Molecular weight
N	Normality (used with NaOH)
N	= A, G, C or T (in DNA base sequence)
OD	Optical density
PEG	Polyethylene glycol
Pu	Purine (= A or G, in DNA base sequence)
Py	Pyrimidine (= C or T, in DNA base sequence)
RNA	Ribonucleic acid
RNase	Ribonuclease A
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
T	Thymine (in DNA base sequence)

Tc^R	Tetracycline resistance
Tris	Tris(hydroxymethyl)aminoethane
UV	Ultra violet light
V/cm	Volt per cm
v/v	Volume to volume
w/v	Weight to volume

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Definition and progress in the study of plasmids

The term 'plasmid' was coined by Lederberg (1952) to connote all extrachromosomal genetic elements which are capable of stable autonomous replication in their host cells, that is, which are independent replicons (Jacob, Brenner and Cuzin, 1963).

The first plasmid known was the sex factor, F, of *Escherichia coli* K12 (Hayes, 1952; Lederberg, Cavalli and Lederberg, 1952), which confers on the host cell the ability to conjugate and to transfer the plasmid and the host chromosome (separately or together) to other cells. Its presence in the cell was demonstrated solely by genetic experiments and progress in the elucidation of its structure and size was slow. However, since 1960, there has been an explosive expansions of our understanding of the operation and structure of the genetic material, including plasmids, at the molecular level, and many different types of plasmids have since been discovered in both prokaryotic and eukaryotic cells (Broda, 1979). This rapid progress was due to a number of reasons:

i) In contrast to the F plasmid, many transmissible plasmids were first discovered not by their ability to promote their own transfer or the transfer of chromosomal DNA via conjugation, but by a variety of easily identifiable new phenotypic traits conferred on their host cells, such as the ability to produce bacteriocin or to resist antimicrobial agents. Likewise, many nontransmissible plasmids

have been discovered.

ii) Initially, plasmids were identified by genetic methods. However, as a result of the development of important technical advances, in addition to the genetic methods, it became possible to study plasmids by a variety of less time-consuming physical, chemical and electromicroscopic techniques. These comprise equilibrium density centrifugation, electromicroscopic analysis, heteroduplex mapping, denaturation mapping, restriction endonuclease mapping, and acrylamide and agarose gel electrophoresis.

iii) The realisation that plasmids are composed of DNA (Lavallé and Jacob, 1961; Driskell and Adelberg, 1963) and exist mainly in the supercoiled ccc form (Roth and Helinski, 1967; Helinski and Clewell, 1971), coupled with the fact that many new techniques are readily available for the isolation of ccc DNA molecules, allow plasmids to be identified physically even before there is any genetic reason to suspect their existence. In this way many plasmids including those without any known function have been identified. This would not have been possible if genetic methods had to be relied upon.

iv) It has become increasingly evident that many of the special characteristics exhibited by bacterial strains which are of importance in medicine, agriculture, industry and the environment are often plasmid-determined. Such characteristics include (1) a variety of virulence properties, such as exotoxins, haemolysin, resistance to antibiotics and the lytic activity of serum (animal pathogens) and tumour-formation activity (plant pathogens), (2) the ability of

nitrogen-fixing *Rhizobium* strains to nodulate roots of legumes, (3) antibiotic production by *Streptomycetes*, and (4) the ability of certain strains of *Pseudomonas* and related organisms to detoxify a range of deleterious organic environmental pollutants, including xenobiotics (Timmis and Pühler, 1979). As a result, concerted effort has been made to identify and isolate more plasmids of special functions.

v) With the advent of the new technology of recombinant DNA (Sinsheimer, 1977), plasmids have become indispensable vehicles for the isolation of specific sequences of DNA, and also convenient tools for the study of DNA mechanisms at the molecular level. More and more plasmids are being investigated in a wide range of organisms in order to study them in different cellular environments and to look into the possibilities of establishing novel host-vector cloning systems.

vi) The recombinant DNA technology has catapulted the science of molecular genetics from the purely academic level to an exciting booming multimillion-dollar industrial level (The Economist, 1978; Wade, 1979, 1980), resulting in more money being made available for further advancement of the science with concomitant expansion in the number of groups working in this field. This exciting technology also brought about the existence of a number of new journals (*Nucleic Acids Research*, *Gene*, *Plasmid*, *Journal of Molecular & Applied Genetics*, *Molecular & Cellular Biology*) in the last decade to accomodate the fast-growing number of publications in this field.

All these factors have contributed, in one way or another, to the rapid progress in the study of plasmids.

1.2 Basic properties of plasmids

Plasmids are widely distributed throughout the prokaryotes. They vary in size from close to 1 x 10^6 daltons to greater than 200 x 10^6 daltons, and are generally dispensable (Broda, 1979).

The distinctive feature of plasmids is their physical separation from the chromosome of the host cell and their stable maintenance in this extrachromosomal state. Certain plasmid elements, designated episome (Jacob and Wollman, 1958a), have the additional property of reversibly integrating with the host chromosome.

Plasmids can be conveniently classified into one of two major types, depending upon whether or not they carry a functional set of genes, called the *tra* genes (Willetts, 1972, 1980), that promote bacterial conjugation (Table 1). The first major class of plasmids are the conjugative or self-transmissible plasmids. These plasmids possess genes which are concerned with their own autonomous replication as well as the *tra* genes that promote bacterial mating. They control the establishment in the host cell of a 'donor state' (through such physical features as sex pili) to provide a mechanism of conjugation that permits their transfer from the host cell into another cell, the recipient, which is subsequently converted into a donor. Because most of the conjugative plasmids can also promote the transfer of other genetic material, either chromosomal or plasmid, they are frequently known as sex, or fertility, factors (Clark and Adelberg, 1962;

TABLE 1

CONJUGATIVE AND NONCONJUGATIVE PLASMIDS

Plasmid Class	Genetic Regions of Plasmid Element		Plasmid Type
nonconjugative	autonomous replication	colicin production	colinogenic (Col) plasmids
		antibiotic resistance	R plasmids
conjugative (sex factors)	autonomous replication and transfer (<u>tra</u>)	regions of bacterial chromosome	F-prime plasmids
		colicin production	colicinogenic (Col) plasmids
		antibiotic resistance	R plasmids
		enterotoxin production	Ent plasmids

Novick, 1969).

These conjugative plasmids are most commonly found among the enteric and related Gram-negative bacteria. However, recently they have also been described in a number of the Gram-positive species: Streptococcus faecalis (Jacob and Hobbs, 1974; Jacob, Douglas and Hobbs, 1975; Dunny and Clewell, 1975), S. lactis (Gasson and Davies, 1980; Walsh and McKay, 1981), S. pneumoniae (Smith, Shoemaker, Burdett and Guild, 1980), oral streptococci (LeBlanc, Hawley, Lee and St. Martin, 1978), group B Streptococcus (Hershfield, 1979), group F Streptococcus and S. avium (Gibson, Chace, London and London, 1979), group N streptococci (McKay, Baldwin and Walsh, 1980), and Streptomyces coelicolor (Hopwood and Wright, 1976; Bibb, Freeman and Hopwood, 1977).

The second major class of plasmids are the nonconjugative or nonself-transmissible plasmids, which have genes that are essential for their own autonomous replication but not genes for the conjugal transfer of the plasmid or the host chromosome. They are therefore not able to set up the 'donor state' in the host cell and require either a conjugative plasmid or a transducing phage for their transfer in natural cell populations.

In addition to the genes mentioned, both conjugative and nonconjugative plasmids may also possess other genes which confer distinct phenotypes on their host cells (Table 2). If the genes are involved in the production of colicin (extracellular antibiotic protein), the plasmid is designated a colicinogenic, or Col,

TABLE 2

Phenotypic traits exhibited by plasmid-carried genes

Antibiotic production

Antibiotic resistance

Bacteriocin production

Enterotoxin production

Fertility

Haemolysin production

Heavy-metal resistance

Host-controlled restriction and modification

Hydrogen sulphide production

Induction of plant tumours

Metabolism of aromatic compounds

Sugar fermentation

UV resistance

plasmid. If the additional gene(s) determines resistance to one or more antibiotics, then the plasmid is designated an antibiotic resistance, or R, plasmid (Mitsuhashi, 1979). If the sex factors incorporate fragments of bacterial chromosome into their structure, they are designated F-prime (F') plasmids (Adelberg and Burns, 1960; Hirota and Sneeth, 1961). Plasmids to which phenotypic traits have not yet been identified are called cryptic plasmids.

Not all plasmids can be stably co-inherited in the same host cell. Two distinct plasmids that can be stably co-inherited in dividing bacteria are said to be compatible, whereas those pairs of plasmids that cannot are termed incompatible. On this basis, plasmids are classified into various incompatibility groups. About 24 incompatibility groups have been described for plasmids of Gram-negative bacteria and 7 for a number of the plasmids of *Staphylococcus aureus* (Novick, Clowes, Cohen, Curtiss, Datta and Falkow, 1976). In general, any member of a particular incompatibility group cannot stably co-exist in a bacterial cell with any other member of that same group, but it can stably co-exist with any member of a different incompatibility group.

Plasmids can also be categorised on the basis of their copy number per cell. Different plasmids are maintained at different copy numbers, ranging from 1 to 50 plasmid copies per genome equivalent, in a particular host bacterium grown under defined conditions, suggesting a different mode of replication control. Relaxed plasmids are maintained as multiple copies per cell and are thought

to be under relaxed control of replication; whereas stringent plasmids are maintained at a limited number of copies per cell and are thought to be under stringent control of replication (Novick et al., 1976). The replication of stringent plasmids is of necessity coupled to chromosome replication, hence their low copy number. Generally, conjugative plasmids are of a relatively high molecular weight and are present as 1-3 copies per chromosome, whereas non-conjugative plasmids are of low molecular weight and present as multiple copies per chromosome (Table 3). An exception is the conjugative plasmid R6K which has a molecular weight of 25×10^6 and is maintained as a relaxed plasmid (Kontomichalou, Mitani and Clowes, 1970). However, R6K, unlike plasmids ColE1, RSF1030 and Cl.DF13, does not continue to replicate in the presence of the protein synthesis inhibitor chloramphenicol. In the case of the ColE1 plasmid, continued replication of the plasmid in the presence of chloramphenicol results in the accumulation of 1,000 - 2,000 copies of the supercoiled DNA form of this plasmid per cell (Bazaral and Helinski, 1970: Clewell, 1972; Hershfield, Boyer, Yanofsky, Lovett and Helinski, 1974).

The mode of replication of plasmids in the host cell was shown by Bazaral and Helinski (1970) to be semi-conservative (Meselson and Stahl, 1958). Most plasmids appear to have a unique initiation point (origin of replication). Elongation from this origin may be unidirectional (e.g. ColE1, Inselburg, 1974; Lovett, Katz and Helinski, 1974; Tomizawa, Sakakibara and Kakefuda, 1974; and RK2,

TABLE 3

STRINGENT AND RELAXED NATURALLY OCCURRING PLASMIDS

Plasmid	Size ($\times 10^6$ d)	Conjugative	No. copies per chromosome	Phenotype
ColE1	4.2	no	10 - 15	Colicin E1 production
RSF1030	5.6	no	20 - 40	Ampicillin resistance
CloDF13	6.0	no	10	Cloacin production
R6K	25	yes	13 - 38	Ampicillin and streptomycin resistance
F	62	yes	1 - 2	-
R1	65	yes	1 - 3	Multiple drug resistance
R6	65	yes	1 - 3	Multiple drug resistance
EntP307	65	yes	1 - 3	Enterotoxin production

Meyer and Helinski, 1977), or bidirectional (e.g. mini-F, Eichenlaub, Figurski and Helinski, 1977). Some plasmids have been reported to have two sites at which initiation occurs (e.g. R6K, Crosa, Luttropp, Heffron and Falkow, 1975; Lovett, Sparks and Helinski, 1975; and NR1, Perlman and Rownd, 1976). Initiation from the two origins in R6K is of particular interest since it is sequentially bidirectional (Crosa et al., 1975; Lovett et al., 1975).

Once initiation has occurred, the elongation process proceeds the same way in all DNA replicons investigated, and involves RNA-primed discontinuous synthesis on at least one of the strands (Inselburg and Oka, 1975; Katz, Williams, Sato, Leavitt and Helinski, 1977). Movement of the replication fork continues until terminated either by meeting another replication fork proceeding in the opposite direction, returning to the origin of replication or arriving at a specific terminus region of DNA (Lovett et al., 1975) that prevents or impedes further elongation.

Using in vivo and in vitro recombinant DNA techniques, miniplasmids containing the minimal DNA fragment required for autonomous replication ('basic replicon') have been derived from a number of plasmids (Timmis, Cabello and Cohen, 1975; Kollek, Oertel and Goebel, 1978: Kolter and Helinski, 1978; Taylor and Cohen, 1979). The nucleotide sequence of a number of functional replication origins (e.g. ColE1-type plasmid pBR322, Sutcliffe, 1979; R6K, Stalker, Kolter and Helinski, 1979; R1, Oertel, Kollek, Beck and Goebel, 1979; and R100, Rosen, Ohtsubo and Ohtsubo, 1979) have been

determined. Fragments of R6K containing the replication terminus region have also been isolated (Kolter and Helinski, 1978).

Although available data on plasmid DNA synthesis requirements are incomplete, they suggest that plasmid replication is largely dependent upon host-coded DNA synthesis functions. Thus far, the products of dnaC (initiation of replication), and the dnaB and dnaG genes (progeny chain elongation) have been shown to be required for plasmid replication, as have DNA polymerase III, RNA polymerase, and the DNA gyrase enzyme (Timmis, Cohen and Cabello, 1978).

Although plasmids are dependent on a number of host functions for their replication, they do retain overall control of this process, and specify some of the replication functions: such as the initiation of plasmid replication thus controlling the plasmid copy number (Nordström, Ingram and Lundbäck, 1972; Burger, Steinbauer, Röllich, Kollek and Goebel, 1981), and plasmid incompatibility (Uhlin and Nordström, 1975; Burger *et al.*, 1981).

The mechanism for the control of plasmid DNA replication remains unclear although several theories exist. They may be grouped into two main categories: one suggesting positive and the other suggesting negative control of replication. In the positive-control model (Jacob *et al.*, 1963), copy number depends upon the number of available membrane attachment sites. Only when new sites become available can replication occur. It

is postulated that each replicon carries a specific replicator region on which a positive effector (initiator) acts to trigger DNA replication. However, the frequently discussed alternative negative-control model of Pritchard, Barth and Collins (1969) proposes that shortly after initiation of replication, a repressor protein is synthesized and it prevents a second round of initiation until the cell volume has increased sufficiently for the repressor concentration to fall below a critical level.

1.3 Isolation of plasmid DNA

From 1952, when F was discovered, until 1959, when the F' state was recognized, the F plasmid was known only as an abstract 'element' and was studied solely by genetic experiments. Its structure and size were unknown. It was assumed to be composed of DNA only by analogy with λ which F resembles in its ability to attach reversibly to the bacterial chromosome and to incorporate chromosomal segments. However, this was soon confirmed when Lavallé and Jacob (1961) and Driskell and Adelberg (1963) showed that F could incorporate ^{32}P-labelled phosphate and transfer it to an F^- recipient, where decay of the unstable isotope led to the eventual loss of the transferred F.

The first direct demonstration of the physical nature of bacterial plasmids came from the work of Marmur, Rownd, Falkow, Baron, Schildkraut and Doty in 1961. They transferred an F'*lac* plasmid from *E. coli* into a strain of non-lactose-utilizing

Serratia marcescens to obtain lactose-utilizing derivatives. Isolation of the DNA from these derivatives by analytical density gradient ultracentrifugation in CsCl showed the presence of two distinct classes of DNA, one having a higher GC content than the other. The former corresponded to the DNA of S. marcescens (58% GC) and the latter to E. coli (50% GC). This provided evidence that plasmid exists as a separate entity from the bacterial chromosome and is composed of DNA. Later, analogous experiments were performed to demonstrate the extra-chromosomal, autonomous nature of the R plasmids (Falkow, Citarella, Wohlhieter and Watanabe, 1966; Rownd, Nakaya and Nakamura, 1966). The extra-chromosomal nature of ColE1 plasmid in Proteus mirabilis was also physically demonstrated by DeWitt and Helinski (1965) by separating it from the Proteus chromosomal DNA in a CsCl density gradient.

In the early experiments, plasmid DNA was isolated as linear molecules because the method used then for its isolation usually led to its breakage into fragments. However, with the development of gentler methods of releasing DNA from cells, plasmids were shown by electron microscopy to be supercoiled ccc molecules (Roth and Helinski, 1967; Freifelder, 1968; Hickson, Roth and Helinski, 1968).

This configuration confers on the plasmids a number of unique properties, such as their decreased intrinsic viscosity, decreased sensitivity to shear, increased resistance to alkali

and thermal denaturation, decreased binding to intercalating dyes at saturating levels of the dyes in buoyant solutions of CsCl, preferential partition into an aqueous phase at acid pH and low ionic strength, and unique electrophoretic mobilities in an agarose gel matrix (Helinski and Clewell, 1971; Aaij and Borst, 1972; Meyers, Sanchez, Elwell and Falkow, 1976; Zasloff, Ginder and Felsenfeld, 1978).

These properties have been extensively exploited to develop methods to isolate and prepare large amounts of pure plasmid DNA molecules from a variety of bacterial cells.

Basically, the procedures involve first the gentle lysis of bacterial cells, using lytic enzymes (such as lysozyme, Clewell and Helinski, 1969; lysostaphin, Novick and Bouanchaud, 1971; and TBL-1, Horinouchi, Uozumi, Beppu and Arima, 1977) and ionic or non-ionic detergents (such as SDS, Brij 58, sodium deoxycholate, sarkosyl and Triton X-100, Clowes, 1972). The lysate is then treated to enrich the ccc plasmid DNA molecules by selective removal of chromosomal DNA. Currently, many enrichment procedures are used and involve one or a combination of the following steps:

i) The selective precipitation of chromosomal complexes by high gravity centrifugation to prepare a 'cleared lysate' (Clewell and Helinski, 1969).

ii) The preferential precipitation of high molecular

weight chromosomal DNA by SDS in the presence of a high concentration of salt (Guerry, LeBlanc and Falkow, 1973).

iii) Selective shearing and then denaturation of linear chromosomal DNA using alkali (Cohen and Miller, 1969; Sharp, Hsu, Ohtsubo and Davidson, 1972; Chassy, Gibson and Giuffrida, 1976; Currier and Nester, 1976; Hansen and Olsen, 1978) or heat (Ohlsson, Hentschel and Williams, 1978).

iv) Selective concentration of ccc plasmid molecules by hydroxyapatite chromatography (Colman, Byers, Primrose and Lyons, 1978; Shoyab and Sen, 1979) or PEG 6000 (Humphreys, Willshaw and Anderson, 1975).

v) Selective removal of denatured linear DNA with nitrocellulose (Cohen and Miller, 1969; Sharp *et al*., 1972; Palchaudhuri and Chakrabarty, 1976) or phenol (Chassy *et al*., 1976; Currier and Nester, 1976).

After the enrichment step, the ccc DNA is separated from the remaining circular and linear DNA by alkaline or neutral sucrose gradient sedimentation (Freifelder, 1970; Freifelder, Folkmanis and Kirschner, 1971; Hughes and Meynell, 1977; El-Gewely and Helling, 1980), or CsCl-dye equilibrium density centrifugation (CsCl-ethidium bromide, Radloff, Bauer and Vinograd, 1967; CsCl-propidium diiodide, Hudson, Upholt, Devinny and Vinograd, 1969; CsCl-Hoechst dye 33258, Fennewald, Prevatt, Meyer and Shapiro, 1978; CsCl-DAPI, Williamson and

Fennell, 1975). During the CsCl-EtBr equilibrium density centrifugation, the ccc DNA, because of its supercoiled configuration, binds less dye than the circular and linear duplex DNA molecules at saturating levels of the intercalating dye. As dye-binding to DNA reduces the buoyant density of the duplex, the ccc DNA bands at a higher density than circular and linear DNA. Whereas separation of the ccc DNA in sucrose gradient sedimentation is based on the fact that ccc molecules sediment more rapidly than circular and linear molecules of the same mass due to their more compact form as a result of supercoiling.

Currently, the purification of ccc plasmid molecules from their host cells is expedited and simplified by the development of many rapid extraction procedures. Again these procedures take advantage of the unique properties associated with the supercoiled ccc configuration of the plasmid molecules. After extraction, detection of the ccc plasmid molecules is based on their unique electrophoretic mobilities in an agarose gel matrix (Aaij and Borst, 1972; Meyers *et al.*, 1976; Willshaw, Smith and Anderson, 1979).

These methods can be grouped into two main categories:

1) The host bacterial cells are gently lysed using lytic enzyme and detergent, or detergent alone, or phenol. The lysate is then used directly for agarose gel electrophoresis, or is heated prior to electrophoresis (Barnes, 1977; Telford, Boseley,

Schaffner and Birnstiel, 1977; Eckhardt, 1978; Ohlsson *et al.*, 1978; Wilson and Baldwin, 1978; Wilson, Totten and Baldwin, 1978; Hepburn and Hindley, 1979; Sherratt, 1979; Klein, Selsing and Wells, 1980; Macrina, Wood and Jones, 1980; Welch and Macrina, 1981).

2) The host cells are gently lysed using lytic enzyme and detergent or detergent alone. The lysate is then treated with alkali to bring about denaturation of DNA molecules, followed by neutralization to selectively renature the ccc plasmid DNA, which is subsequently detected by agarose gel electrophoresis (Birnboim and Doly, 1979; LeBlanc and Lee, 1979; Austen and Trust, 1980; Imanaka, Fujii and Aiba, 1981; Kado and Liu, 1981; Omura, Ikeda and Tanaka, 1981; Wheatcroft and Williams, 1981).

As a result of these isolation techniques, the literature is burgeoned with reports revealing the presence of extrachromosomal ccc DNA molecules in both prokaryotic and eukaryotic cells. In fact, in many cases, plasmids are first identified physically before there is any genetic reason to suspect their existence. Some of them are later related to certain phenotypes while other plasmids remain cryptic.

1.4 Uses of plasmids

Initially, plasmids particularly the sex factors were used to map the bacterial chromosome genetically via the process of

conjugation (Jacob and Wollman, 1958b; Smith and Stocker, 1962). Later, as more plasmids were discovered, they were studied for their own sake to reveal details of their size, structure, replication, incompatibility, transmissibility, ability to mobilize chromosomal DNA, resistance to antibiotics, production of colicins, and other functions, as well as their evolution in natural populations. They also became convenient tools and ideal models for the study of DNA mechanisms at the molecular level, such as the control of DNA replication, the intra- and inter-generic exchange of genetic material between strains via conjugation.

In the last 12 years, with the emergence of the new technology of recombinant DNA/genetic engineering (Sinsheimer, 1977), a new dimension was added to the uses of plasmids which increased their importance phenomenally. The recombinant DNA technology permits the transfer of genetic material between widely divergent species and it involves four essential elements (Cohen, 1975) :

i) a method of breaking and joining DNA molecules derived from different sources;

ii) a suitable gene carrier (vector) that can replicate both itself and a foreign DNA segment linked to it;

iii) a means of introducing the composite DNA molecule, or chimera, into a functional cell; and

iv) a method of selecting from a large population of cells a clone of recipient cells that has acquired the molecular chimera.

Plasmids, as can be seen from below, possess properties which allow genetic engineering to be carried out easily, and they play an indispensable role in the recombinant DNA technology.

i) Cutting and joining DNA molecules

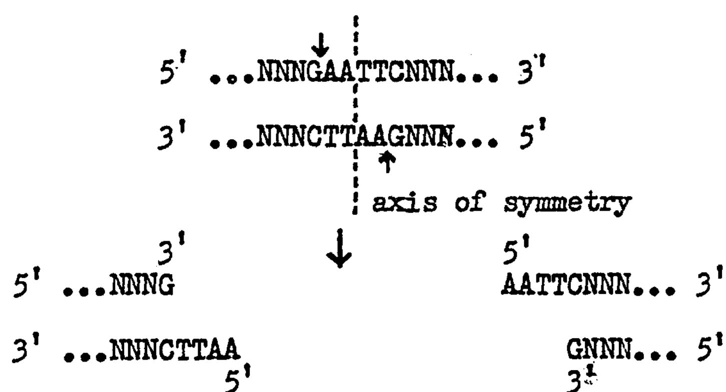
Central to the success of recombinant DNA technology is the discovery of host-controlled DNA restriction and modification systems in bacterial cells, which are mediated by two enzymes, a restriction endonuclease and a modification methylase (Arber, 1965; Boyer, 1971; Meselson, Yuan and Heywood, 1972). Both enzymes are believed to recognize the same substrate, e.g. a sequence of 4-8 nucleotide base pairs in length. The methylase brings about the methylation of two bases, one in each strand of the recognized sequence. The restriction endonuclease cleaves two phosphodiester bonds in the sequence, one in each polynucleotide strand, provided the sequence has not been methylated with the related modification methylase. This explains how a cell can have a site-specific endonuclease without destroying its own chromosome (Boyer, 1974).

There are two classes of restriction endonucleases: type I and type II. The type I restriction endonucleases require Mg^{++}, ATP and S-adenosyl-methionine for endonucleolytic activity on unmodified DNA. Although they recognize specific sites within the DNA, they do not cleave at these sites (Meselson *et al.* 1972; Rosamond, Endlich and Linn, 1979). In contrast, the type II restriction endonucleases require only Mg^{++} for endonucleolytic activity on unmodified DNA and cleave it at, or near, specific sequences, that are usually several nucleotides

long and rotationally symmetrical about the central nucleotide pairs. They are invaluable for cloning DNA because they give rise to discrete DNA fragments of defined length and sequence.

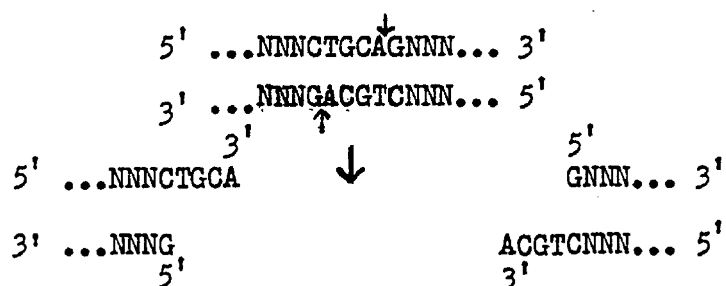
Very many type II restriction endonucleases have now been isolated from a wide range of prokaryotic microorganisms, and the sequences recognized by them determined (Roberts, 1980). In general, they cleave DNA to generate fragments with 5'-phosphate and 3'-hydroxyl groups, belonging to one of the following three types:

a) <u>protruding 5'-ends</u> : e.g.



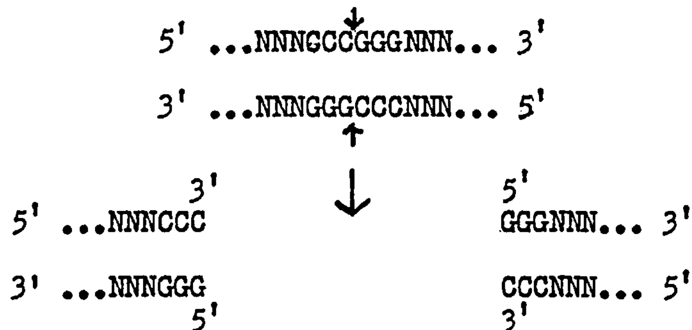
Fragments with single-stranded mutually cohesive or sticky protruding 5'-ends produced by staggered nicks of a type II restriction endonuclease, <u>EcoRl</u>.

b) <u>protruding 3'-ends or recessed 5'-ends</u> : e.g.



Fragments with single-stranded mutually cohesive or sticky protruding 3'-ends produced by staggered nicks of a type II restriction endonuclease, PstI.

c) flush or blunt ends : e.g.



Fragments with no single-stranded cohesive protruding end produced by a type II restriction endonuclease, Sma I. Note that the cleavage sites are along the axis of symmetry.

Endonucleases which introduce staggered nicks (e.g. EcoRl, Pstl) are of particular importance for they produce fragments with complementary single-stranded segments which can be efficiently ligated, even when the fragments are from diverse sources.

Plasmids, like all other DNA molecules, are cut by various type II restriction endonucleases at specific cleavage sites, generating DNA fragments of specific length and termini. Fragments generated from a particular plasmid molecule can be separated by electrophoresis in agarose or polyacrylamide gels. The order of these fragments in the plasmid molecule can subse-

quently be determined to provide a physical cleavage map to fingerprint it as well as to serve as a reference for mapping other functions.

Another method commonly used to generate random DNA fragments for molecular cloning is by means of controlled mechanical shearing (Hogness and Simmons, 1964; Clarke and Carbon, 1975, 1976; Ratzkin and Carbon, 1977).

Having produced DNA fragments, they can then be joined *in vitro* by a number of ways to create artificially recombinant molecules. One of the ways capitalizes on the ability of DNA ligase, either from *E. coli* (Modrich, Anraku and Lehman, 1973) or phage T4 (Weiss, Jacquemin-Sablon, Live, Fareed and Richardson, 1968), to join covalently the annealed short cohesive ends produced by certain type II restriction endonucleases (Mertz and Davis, 1972) (Fig. 1). Another way depends upon the ability of DNA ligase from phage T4-infected *E. coli* to catalyse the formation of phosphodiester bonds directly between blunt-ended fragments (Sgaramella, Sande and Khorana, 1970; Sgaramella and Khorana, 1972). A modification of this method is to join the blunt-ended fragments first to synthetic linker or adaptor molecules which can then be cleaved by specific restriction endonuclease to generate cohesive ends (Bahl, Marians, Wu, Stawinski and Narang, 1976; Scheller, Dickerson, Boyer, Riggs and Itakura, 1977) (Fig. 2).

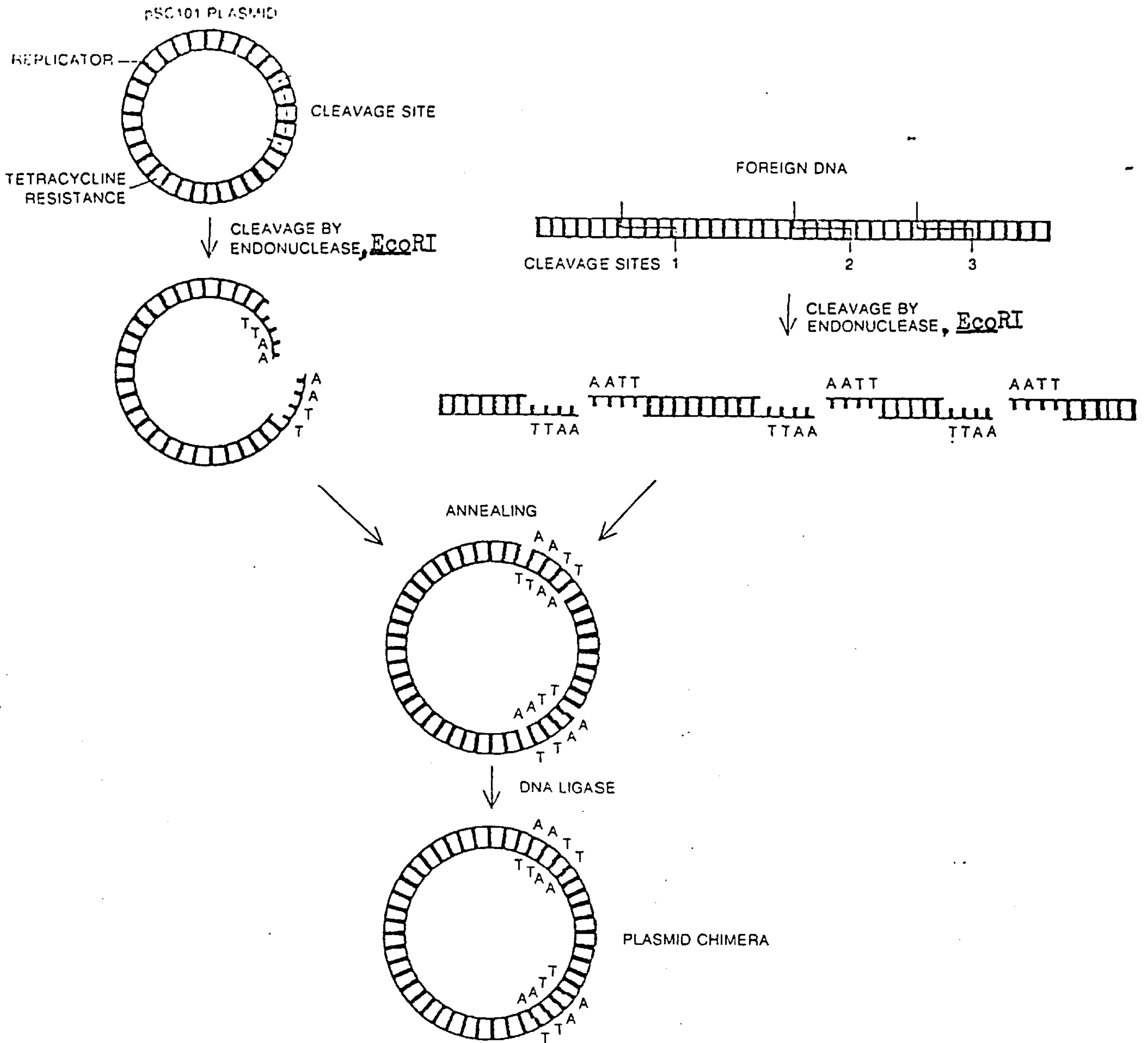


Fig. 1. Joining of the EcoRI - generated cohesive ends by DNA ligase.

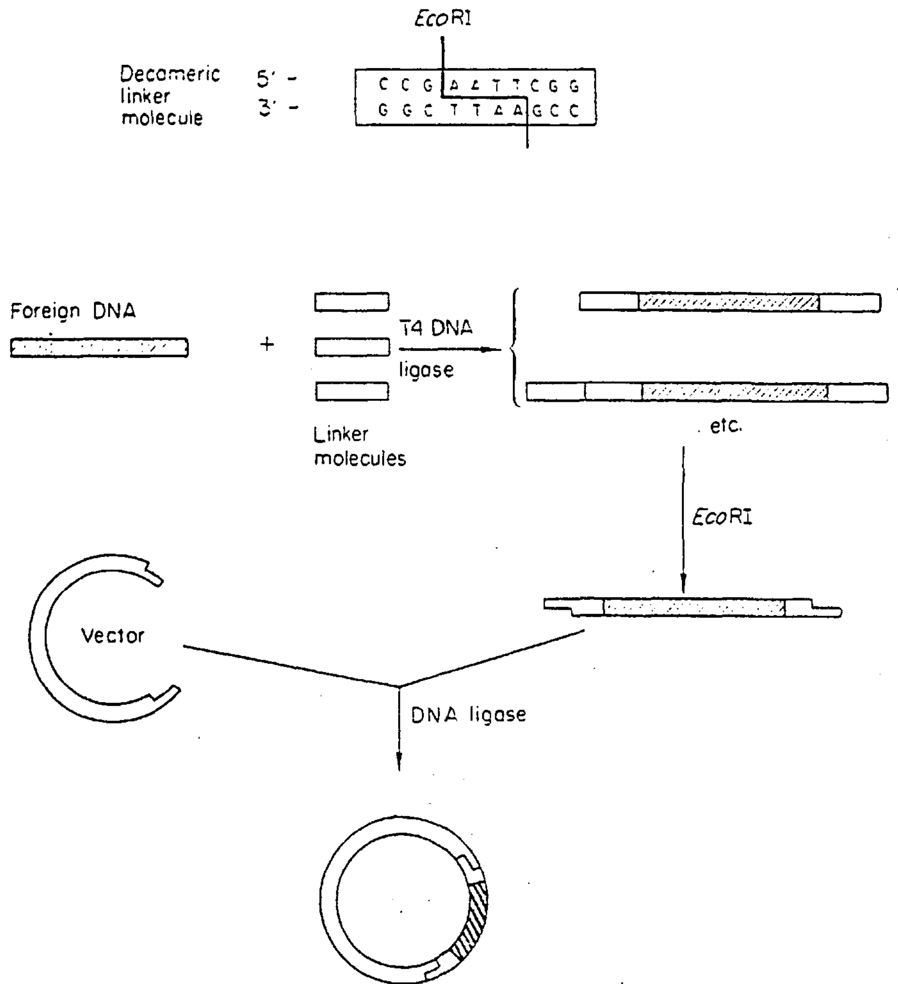


Fig. 2. A decameric linker molecule containing an *Eco* RI target site is joined by T4 DNA ligase to both ends of flush-ended foreign DNA. Cohesive ends are then generated by *Eco* RI. This DNA can then be incorporated into a vector that has been treated with the same restriction endonuclease.

Another method utilizes an enzyme from calf-thymus, terminal deoxynucleotidyl-transferase, to synthesize homopolymeric 3'-single-stranded tails at the ends of fragments, e.g. oligo(dA) sequences at the 3'-ends of one population of DNA molecules, and oligo(dT) sequences at the 3'-ends of another population of DNA molecules. The two types of molecules are mixed and allowed to anneal at their complementary homopolymeric tails (Jensen, Wodzinski and Rogoff, 1971; Jackson, Symons and Berg, 1972; Lobban and Kaiser, 1973) (Fig. 3).

ii) <u>Vectors for genetic manipulation</u>

The commonly used cloning vectors are bacterial plasmids (Bolivar and Backman, 1979; Kahn, Kolter, Thomas, Figurski, Meyer, Remaut and Helinski, 1979) and bacteriophages (Enquist and Sternberg, 1979; Hohn, 1979). Both these vector molecules are autonomous replicons and can be cleaved by type II restriction endonucleases at sites not affecting their ability to replicate in their host cells. The fragments generated are then ligated by one of the previously mentioned methods to other passenger DNA fragments which are produced by one of the following means: a) restriction endonuclease digestion, b) controlled shearing, c) enzymatic copying of RNA (i.e. cDNA) (Goodman and MacDonald, 1979), and d) chemical synthesis (Brown, Belagaje, Ryan and Khorana, 1979).

In this way the passenger DNA fragments which usually lack

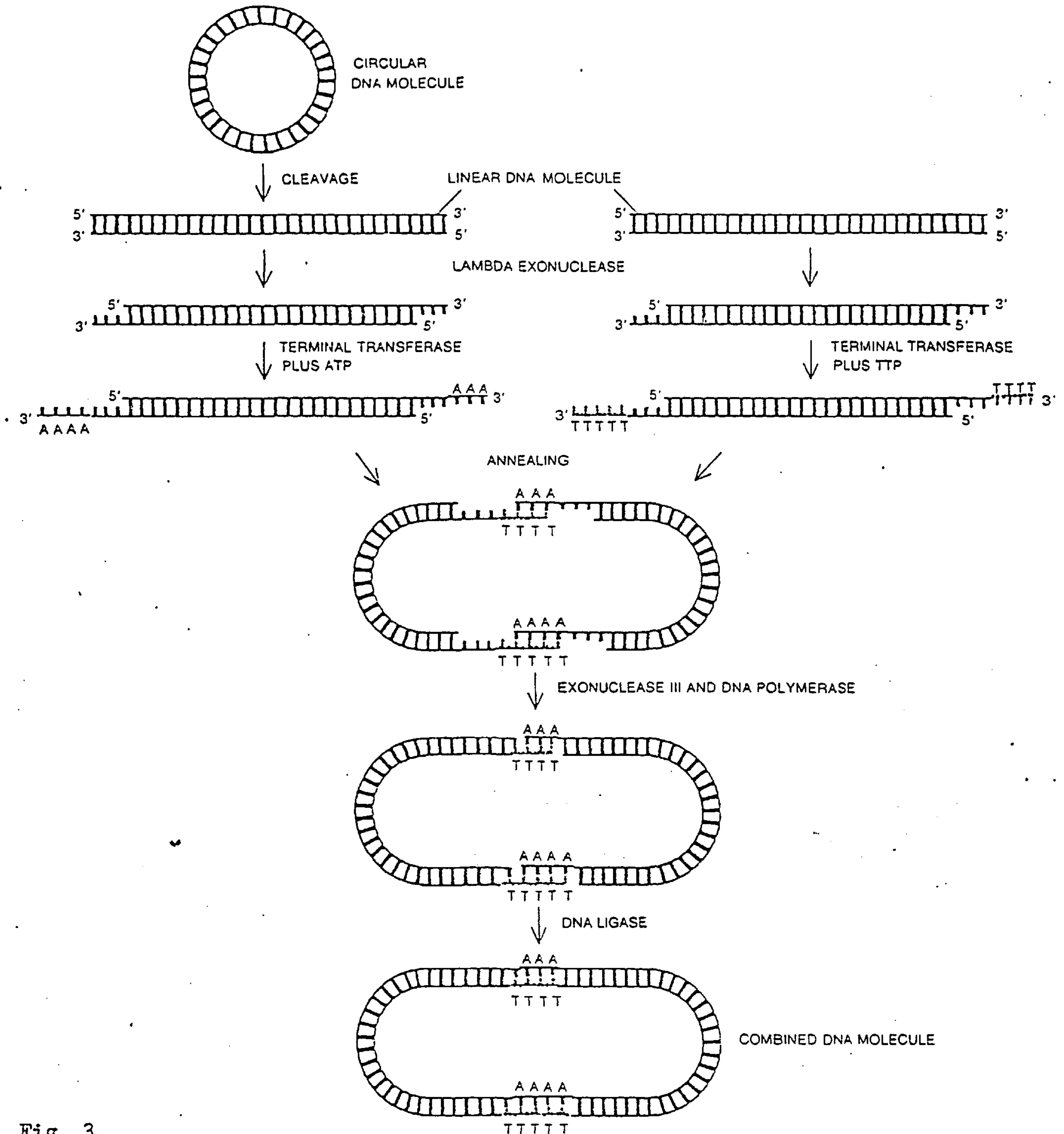


Fig. 3.

TERMINAL-TRANSFERASE procedure for joining DNA molecules involves a number of steps, each dependent on a different enzyme. If one of the molecules to be joined is a closed loop, it must first be cleaved. The linear molecules are treated with lambda exonuclease, an enzyme that cuts nucleotides off the 5′ end of DNA strands (the end with a phosphate group on the No. 5 carbon). Then specific nucleotides are added to the 3′ end (the end with an OH group on the No. 3 carbon) by the action of the enzyme terminal transferase. One DNA species is supplied with adenosine triphosphate (ATP), the other with thymidine triphosphate (TTP), so that *A* nucleotides are added to one species and complementary *T* nucleotides to the other. When the two species are mixed, the complementary bases pair up, annealing the molecules. Nucleotides and the enzymes DNA polymerase and exonuclease III are added to fill gaps and DNA ligase is added to seal the DNA backbones. The result is a double molecule composed of two separate DNA segments.

the capacity for self-replication are integrated into a replicon to form a chimeric molecule and replicate passively together with it in a suitable host.

iii) Entry of the recombinant DNA molecules into cells

Transformation refers to the process by which naked DNA is introduced into a recipient cell, resulting in a heritable change (Avery, MacLeod and McCarty, 1944). Although transformation occurs under normal growth conditions in a number of bacteria, such as *Acinetobacter*, *Bacillus subtilis*, *Diplococcus pneumoniae*, *Haemophilus influenzae*, *Micrococcus radiodurans*, *Neisseria*, and *Streptococcus* (Ravin, 1961; Notani and Setlow, 1974), it does not occur normally in most bacteria.

One of the biggest achievements in the early 1970's is the establishment of procedures to introduce DNA molecules into *E. coli* by *in vitro* means. In 1970 Mandel and Higa discovered the procedure of transfection in which treatment of *E. coli* with calcium salts enabled the bacteria to take up viral DNA. Later, Cohen, Chang and Hsu (1972), using a similar approach, discovered the procedure of transformation in which plasmid DNA was introduced into $CaCl_2$-treated *E. coli* cells. Using this transformation procedure, the first *in vitro* constructed hybrid molecule, containing the plasmid pSC101 and an inserted DNA segment, was introduced into

E. coli cells (Cohen, Chang, Boyer and Helling, 1973; Chang and Cohen, 1974; Morrow, Cohen, Chang, Boyer, Goodman and Helling, 1974) (Fig. 4).

Since then, effective plasmid-mediated transformation systems have been developed for a number of species: *Azotobacter vinelandii* (David, Tronchet and Dénarié, 1981), *B. subtilis* (Ehrlich, 1977; Gryczan and Dubnau, 1978; Keggins, Lovett and Duvall, 1978), *Pseudomonas* (Chakrabarty, Mylroie, Friello and Vacca, 1975; Sano and Kageyama, 1977; Nagahari and Sakaguchi, 1978; Bagdasarian, Bagdasarian, Coleman and Timmis, 1979); *Salmonella typhimurium* (Lederberg and Cohen, 1974), *S. aureus* (Lindberg, Sjöström and Johansson, 1972; Lindberg and Novick, 1973), and *Streptococcus* (LeBlanc and Hassell, 1976; LeBlanc, Cohen and Jensen, 1978).

Recently, another plasmid-mediated transformation system has been reported for a number of microbial cells involving protoplast/sphaeroplast fusion induced by PEG: *B. megaterium* (Brown and Carlton, 1980; Vorobjeva, Khemel and Alföldi, 1980), *B. subtilis* (Chang and Cohen, 1979), *B. thuringiensis* (Alikhanian, Ryabchenko, Bukanov and Sakanyan, 1981), *Saccharomyces* (Hinnen, Hicks and Fink, 1978), *Schizosaccharomyces pombe* (Beach and Nurse, 1981), *Staphylococcus* species (Götz, Ahrné and Lindberg, 1981), and *S. coelicolor* (Bibb, Ward and Hopwood, 1978).

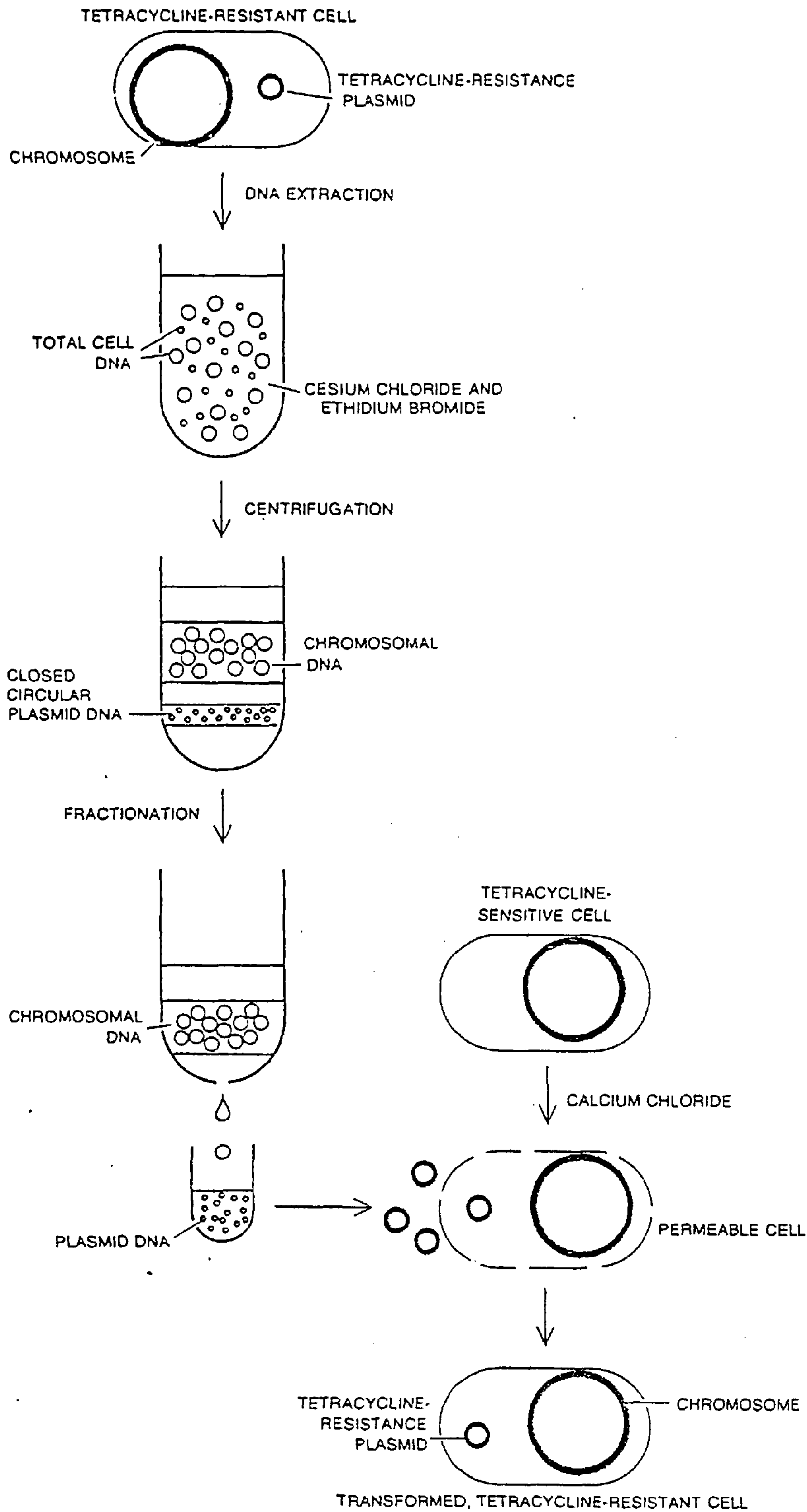


Fig. 4.

PLASMID DNA can be introduced into a bacterial cell by the procedure called transformation. Plasmids carrying genes for resistance to the antibiotic tetracycline (*top left*) are separated from bacterial chromosomal DNA. Because differential binding of ethidium bromide by the two DNA species makes the circular plasmid DNA denser than the chromosomal DNA, the plasmids form a distinct band on centrifugation in a cesium chloride gradient and can be separated (*bottom left*). The plasmid DNA is mixed with bacterial cells that are not resistant to tetracycline and that have been made permeable by treatment with a calcium salt. The DNA enters the cells, replicates there and makes the cells resistant to tetracycline.

Another method for introducing recombinant DNA molecules into *E. coli* cells involves the transduction of recombinant λ and cosmid DNA packaged by *in vitro* means in the λ phage particles (Hohn and Murray, 1977; Sternberg, Tiemeier and Enquist, 1977; Collins, 1979).

Lately, a novel approach to introduce specific DNA sequences into cells is by the use of liposomes as a vector (e.g. into bacterial cells, Fraley, Fornari and Kaplan, 1979; into plant cells, Lurquin, 1979; and into animal cells, Wong, Nicolau and Hofschneider, 1980).

iv) Selection of clones containing recombinant molecules

It is necessary to be able to select, or identify, those cells in a bacterial population which have been transformed, transfected or transduced with the DNA chimeras. A number of methods are available.

a) Genetic methods

1) Selection for the presence of vector

Both plasmid and phage vectors used for cloning experiments normally carry a selectable genetic marker. Plasmid vectors may carry drug resistance, colicinogenic or nutritional markers, and in the case of phage vectors, plaque formation itself is the selectable property. Successfully transformed cells are selected by acquisition of a vector-

determined phenotype, such as immunity to colicin E1 or resistance to an antibiotic. It is important then to identify clones, among a population of the transformed cells, in which the plasmid vector contains inserted DNA.

To ensure insertion of the passenger DNA molecules to the vector DNA, particularly in cases where the vector and the passenger DNA molecules have homologous cohesive ends, one frequently used method is to treat the endonuclease-digested vector molecules with bacterial alkaline phosphatase at 65°C to remove the 5'-terminal phosphates from their ends. This prevents recircularization of the vector DNA and ensures that the majority of the transformants contain hybrid plasmids (Ullrich, Shine, Chirgwin, Pictet, Tischer, Rutter and Goodman, 1977).

Similarly, the use of the enzyme terminal deoxyribonucleotidyl-transferase (Section 1.4.i, Fig. 3) to add complementary single-stranded homopolymers (e.g. poly(dA) and poly(dT) homopolymers) to two different DNA fragments ensures that fragments with complementary homopolymeric tails anneal to each other (e.g. poly(dA.dT) annealing) and not to themselves. Transformants arising from such ligation mixtures consist almost entirely of hybrid molecules, making selection relatively easy.

One method commonly used to detect plasmid vectors

carrying inserted DNA is to isolate the vectors from the transformed cells and size them physically by agarose gel electrophoresis. Plasmid vectors having an inserted DNA fragment would be larger than the nonrecombinant parental vector (Michel, Palla, Niaudet and Ehrlich, 1980).

Another method for identifying clones carrying plasmids with inserted DNA relies on the inactivation of a gene upon the insertion of foreign DNA into that gene, i.e. insertional inactivation (Timmis, Cabello and Cohen, 1974). Many plasmids determine more than one phenotype, and insertion of a passenger DNA fragment at some sites in the plasmids inactivates a gene determining one of their phenotypes. Clones harbouring recombinant plasmids can be rapidly identified by the absence of a vector-associated phenotype (Fig. 5). Some of the specially constructed plasmid cloning vectors having this insertional marker inactivation feature are given in Table 4.

In experiments involving insertional inactivation, if the inactivated gene determines resistance to a bacteriostatic antibiotic such as tetracycline, clones harbouring inserted DNA may be selected for antibiotic sensitivity via a cycloserine enrichment procedure (Bolivar and Backman, 1979).

Recently, Bochner, Huang, Schieven and Ames (1980) and Maloy and Nunn (1981) described a technique for selecting directly tetracycline-sensitive clones from a predominantly

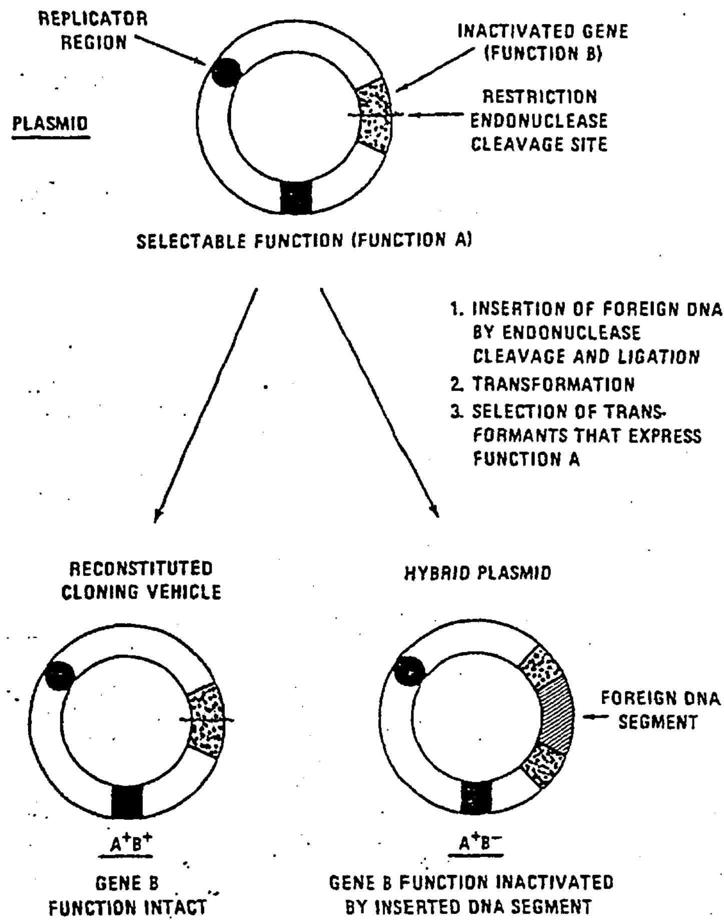


Fig. 5. The principle of insertional inactivation.

TABLE 4

PLASMID CLONING VECTORS WITH SITES FOR INSERTIONAL INACTIVATION

Plasmid	Genetic markers	Sites at which insertion inactivates a marker (indicated in parenthesis)	Reference
pBR322	Tc^R, Ap^R	*BamHI* (Tc^R), *HindIII* (Tc^R), *SalI* (Tc^R), *PstI* (Ap^R), *PvuI* (Ap^R)	Bolivar *et al.*, 1977
pBR325	Cm^R, Tc^R, Ap^R	*EcoRI* (Cm^R), *HindIII* (Tc^R), *BamHI* (Tc^R), *SalI* (Tc^R), *PstI* (Ap^R), *PvuI* (Ap^R)	Bolivar, 1978
RSF2124	Colicin production, ImmEl	*EcoRI* (CP), *SmaI* (CP)	So, Gill and Falkow, 1975
pML21	ImmEl, Km^R	*HindIII* (Km^R)	Timmis, Cabello and Cohen, 1978
pHV23	Ap^R, Cm^R, Tc^R	*PstI* (Ap^R), *PvuI* (Ap^R), *KpnI* (Tc^R)	Michel *et al.*, 1980

Ap^R, Cm^R, Km^R, Tc^R: resistance to ampicillin, chloramphenicol, kanamycin and tetracycline respectively; ImmEl : immunity to colicin El; CP : colicin production

tetracycline-resistant population of bacterial cells. This provides a rapid means of selecting positively hybrid plasmid vectors inactivated at the tetracycline gene by the insert.

There are now a number of artificially constructed plasmid cloning vehicles which allow direct selection of transformants harbouring recombinant plasmids. This is based on insertional inactivation of one function with concomitant insertional activation of another easily selectable function, e.g. pKN80 (insertional inactivation of a lethal function, Schumann, 1979)*, pTR262 (insertional inactivation of the λ repressor gene and activation of the Tc^R gene, Roberts, Swanberg, Poteete, Riedel and Backman, 1980), and pUR2 (insertional inactivation of the α-peptide and activation of the β-galactosidase gene, Rüther, 1980).

2) Selection of the inserted sequences

The selection of plasmids carrying a specific fragment (or an arrangement of fragments) is readily accomplished if that fragment determines a selectable phenotype. In this way, various drug resistance determinants, nutritional markers, and phage immunity genes have been cloned. Cloned nutritional markers from a number of microorganisms, such as *E. coli* (Clarke and Carbon, 1975, 1976), yeast (Struhl, Cameron and

* pKN80 : insertional inactivation of the phage Mu killing function, allowing the positive selection of hybrid DNA in Mu sensitive *E. coli*.

Davis, 1976; Ratzkin and Carbon, 1977), and *B. subtilis* (Nagahari and Sakaguchi, 1978), have been successfully identified by complementation of the *E. coli* auxotrophic mutations *in vivo*.

In an interesting variation of the usual practice of cloning a DNA fragment on a vector having a replicator and a selectable marker, various groups have used a nonreplicating DNA fragment that determines an antibiotic resistance to clone fragments containing replicators (Timmis, Cabello and Cohen, 1975; Lovett and Helinski, 1976).

b) Nucleic acid hybridization methods

Clones carrying a specific DNA fragment in a plasmid vector can be identified by the presence of that fragment's nucleotide sequence. Grunstein and Hogness (1975) have developed a method of releasing, denaturing and immobilizing the DNA of transformed colonies on nitrocellulose filters, and hybridizing radioactive RNA probe to the filters *in situ* to ascertain rapidly which of the colonies contain the specific sequences desired (Fig. 6). With slight modification, this colony hybridization procedure can be applied to phage plaques (Jones and Murray, 1975; Kramer, Cameron and Davis, 1976; Sanzey, Mercereau, Ternynck and Kourilsky, 1976; Benton and Davis, 1977; Woo, 1979), as well as to ^{32}P-labelled DNA probes

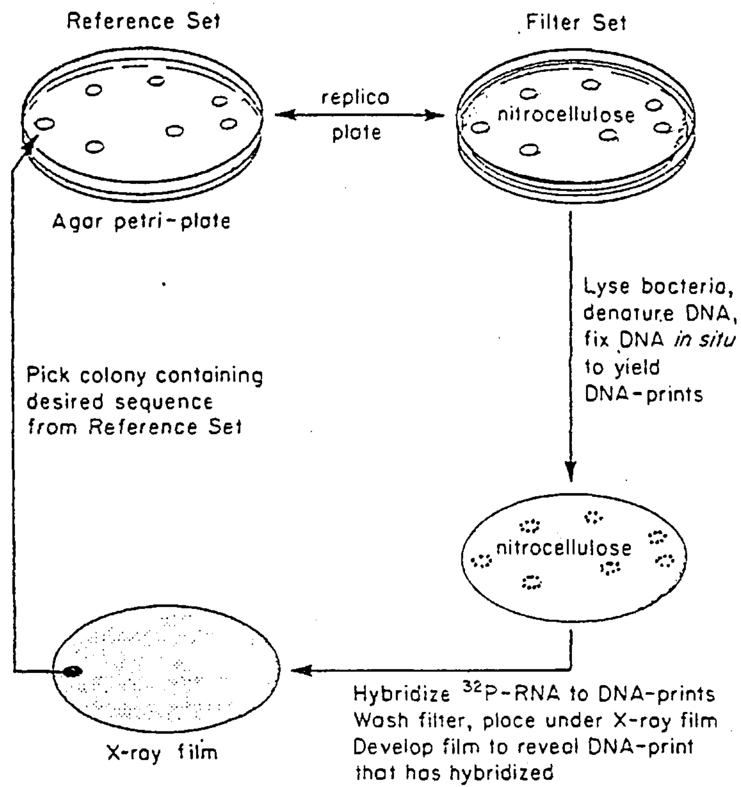


Fig. 6. The rationale of colony hybridization.

conveniently prepared by the process of nick-translation (Rigby, Dieckmann, Rhodes and Berg, 1977). An improved modified method of Grunstein and Hogness (1975) using radioactive DNA or RNA probes was recently described by Hanahan and Meselson (1980).

As the use of a radioactive DNA or RNA probe to detect specific cloned DNA sequences does not require that the sequences are transcribed and translated, the nucleic acid hybridization method has a great advantage in that it can identify those sequences that are not expressed.

c) Immunochemical methods

In some cases where the inserted gene sequences is expressed, the foreign proteins in the recombinant clones can be successfully detected by *in situ* immunoassay methods. The first attempts at immunoassays detected visible immunoprecipitates around the plaques or colonies when the specific antiserum was contained in the growth medium. The sensitivity and rapidity of this screening method has been greatly increased by the use of radioactively labelled antibody probes and a solid phase support for the antibody (Sanzey *et al.*, 1976; Skalka and Shapiro, 1976; Broome and Gilbert, 1978; Ehrlich, Cohen and McDevitt, 1978).

This method does not rely upon complementation or

enzymatic activity, and is sensitive, specific and effective, even if only a small quantity of antigen, or perhaps a fragment of an antigen that still retains antigenic determinants, is produced. It plays a major role in the effort to obtain expression of eukaryotic and certain prokaryotic gene sequences in E. coli, e.g. proinsulin (Villa-Komaroff, Efstratiadis, Broome, Lomedico, Tizard, Naber, Chick and Gilbert, 1978).

All the above mentioned procedures therefore achieve the creation and selection of new genetic combinations which were not possible before the 1970's. Once a desired clone has been identified and isolated, large quantities of the of the recombinant molecule can be prepared, the expression of the passenger DNA studied, the nucleotide sequence of the DNA insert determined, and the gene product amplified and purified (Wu, 1979; Glover, 1980; Old and Primrose, 1980). It is obvious that plasmids play an integral role in this new recombinant DNA technology. Their properties, which include the ability to function as an autonomous replicon, the ease of isolation in large amounts, easily selectable markers, sites for endonuclease digestions, sites for insertion of passenger DNA, sites for insertional inactivation, ability to be amplified to high copy numbers in the host cells, and ability to be transformed into cells, make them ideal vectors for genetic manipulations.

1.5 Alternative host-vector cloning systems

The genetics and physiology of *E. coli* K12 are most comprehensively known, and it is therefore not surprising that the initial cloning experiments were performed in *E. coli* using *E. coli* plasmids as cloning vectors (Cohen *et al.*, 1973). Since then, in the past decade, the components of the *E. coli* cloning systems have been extensively modified by genetic and biochemical manipulations to provide a variety of sophisticated phages, plasmids, and recipient bacterial strains specifically useful for molecular cloning (Wu, 1979). The current *E. coli* host-vector cloning systems have been shown to be extremely reliable for the isolation, amplification and, in many instances, genetic expression of prokaryotic, certain eukaryotic and synthetic DNA fragments.

However, there are a number of potential biohazards and limitations associated with *E. coli* as a cloning host. It is a facultatively anaerobic, Gram-negative, enteric bacterium and is a normal inhabitant of the human and animal intestines. Furthermore most strains produce a lipopolysaccharide endotoxin that is contained in the cell wall. These factors make *E. coli* potentially a hazardous system to work with, especially when it is grown on a large scale

at the industrial level.

In some experiments it is not practicable to use *E. coli* as the host bacterium because it is known to lack some auxiliary biochemical pathways that are essential for the phenotypic expression of certain functions (e.g. toluene degradation, plant pathogenicity). Furthermore, a number of species barriers, such as the nuclease barrier, the replication unit barrier, the transcription barrier, the translation barrier, the proteinase barrier and the intervening gene barrier (Sakaguchi, 1980), are known to exist that prevent the proliferation and expression of foreign DNA introduced into a particular cell.

The use of a different host-vector cloning system would therefore allow an extension of the recombinant DNA technology to systems other than *E. coli*. It would be possible then to use the cloning technology to investigate biological phenomena which might be absent in *E. coli*, but present in the other host. In addition, it would be possible to study the interactions of the same genes with two or more different cellular environments.

Consequently, many organisms are now under intensive investigations in the hope that ultimately some might prove to be good alternative hosts to *E. coli* in certain specific

recombinant DNA experiments (Sherwood and Atkinson, 1981). Some of the alternative host-vector cloning systems currently being developed for genetic manipulations are : *B. subtilis* (Ehrlich, Jupp, Niaudet and Goze, 1978; Young and Wilson, 1978; Young, 1980), *Pseudomonas* (Bagdasarian *et al.*, 1979), *S. cerevisiae* (Fink, Hicks and Hinnen, 1978), and *Streptomyces* (Suarez and Chater, 1980; Thompson, Ward and Hopwood, 1980).

1.6 *Thermus* host-vector cloning system

The establishment of *in vitro* recombinant DNA techniques in an extreme thermophile would be an interesting alternative host-vector system. It would greatly facilitate investigations into the problems of thermostability of macromolecules and of thermophily. It would also provide a convenient system to test whether genes from mesophilic bacteria could function in thermophiles, and if they do, whether products of the genes are converted to heat resistance. This would invariably give us insights into the general problems of microbial evolution and adaptation. Furthermore, an effective cloning system in the extreme thermophiles would provide a means to amplify the production of macromolecules in them, and might be a boon to the fermentation industry.

The extreme thermophiles belonging to the genus Thermus are obligate aerobes, Gram-negative rods, nonmotile, not spore-forming, not parasitic, and grow well only above 45°C with an optimum growth temperature of 60° - 75°C (Williams, 1975; Brock, 1978). Although a cloning system for these thermophiles has not yet been established, they are candidates for experimental host-vector systems which might be safer than the E. coli system.

To establish a system for genetic manipulation in Thermus, it is necessary to develop first a suitable vector, plasmid or bacteriophage, capable of replicating stably in Thermus at temperature up to 75° - 80°C, and then secondly, a transformation system in a suitable Thermus host.

At the outset of this project in October 1977, only one bacteriophage, ØYS40 , isolated from T. thermophilus HB8 (Sakaki and Oshima, 1975) was known. However, it has a limited host range and is not as well characterized as the E. coli phage λ, which is used as a DNA cloning vector in the latter bacterium (Murray, 1978). To be useful as a cloning vector, a detailed genetic and physical knowledge of the phage is required. It is therefore still too early to exploit a Thermus cloning system based on a phage replicon. A plasmid replicon is preferred as the cloning vector.

At that time, in contrast to the studies of plasmids in mesophiles, there was not a single published report of plasmid in thermophilic microorganisms, although later, during the course of this study, five reports were encountered. Four of these reports (Sharp, Bingham, Comer and Atkinson, 1979; Bingham, Bruton and Atkinson, 1979, 1980; and Imanaka, Fujii and Aiba, 1981) are on plasmids from thermophilic bacilli, and the other one (Hishinuma, Tanaka and Sakaguchi, 1978) is on plasmids from _T. flavus_ and _T. thermophilus_.

The ultimate aim of this research is to establish a _Thermus_ DNA cloning system, the development of which has been hindered by the lack of suitable vector replicons and a transformation system. As a first step towards this aim, exploratory experiments were performed to test whether any of the existing plasmids could be used as cloning vectors in _Thermus_. The plasmid RP4, belonging to the promiscuous incompatibility group P, was used as it was shown to have a wide host range and had been successfully transferred to many Gram-negative bacterial species (Datta and Hedges, 1972). Attempts were made to transfer RP4 into _T. aquaticus_ via conjugation and transformation, using the procedures established in _E. coli_ and _Pseudomonas_. However, these preliminary attempts were not successful.

It was reasoned that the realistic approach to this problem would be to look for plasmids originating in *Thermus*, characterize them, and then, if possible, use them to establish a transformation procedure in *Thermus*. Accordingly, experiments were designed :

i) to develop a method to screen for the presence of plasmids in some *Thermus* species : *T. ruber*, and *T. aquaticus* strains isolated from Icelandic hot springs, kindly given by Dr. R.A.D. Williams;

ii) to develop a method to isolate and prepare in large amounts the *Thermus* plasmids;

iii) to characterize the *Thermus* plasmids with respect to antibiotic resistance, bacteriocin production, and restriction endonuclease digestions; and

iv) to construct hybrid plasmid which might be useful in the subsequent development of a transformation procedure in *Thermus*.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Bacterial strains

Bacterial strains used are listed in Table 5.

2.2 Plasmids

Plasmids used in this work are listed in Table 6.

2.3 Materials

Wherever possible, all chemicals used were of Analar grade, or of the highest grade available commercially, obtained from BDH Ltd., Poole, Dorset; Fisons Scientific Apparatus, Loughborough, Leicestershire; James Burrough Ltd., London; May & Baker Ltd., Dagenham; and Sigma London Chemical Co. Ltd., Poole, Dorset. These included amino acids, boric acid, bromophenol blue, cesium chloride, chloramphenicol, chloroform, diethyl ether, DTT, EDTA disodium salt, ethidium bromide, ficoll, glacial acetic acid, D-glucose, isoamyl alcohol, isopropanol, kanamycin sulphate, lysozyme chloride, nitrilotriacetic acid, PEG 6000, SDS, sucrose, sulphafurazole, tetracycline HCl, Tris, Triton-X 100, and other common inorganic chemicals and solvents.

TABLE 5

Bacterial Strains Used

Species and strains	Genotype, if known	Source
Bacillus subtilis		
HVS 49	trypC2 hisH2 tyrA1 aroB2	S.D.Ehrlich
Escherichia coli		
JA221	recA1 leuB6 trypΔE5 hsdR⁻ hsdM⁺ lacY; C600	L.Clarke & J.Carbon
SK1592	F⁻ gal thi T1ʳ endA sbcB15 hsdR4 hsdM⁺	S.R.Kushner
Thermus aquaticus Icelandic strains		
B1		R.A.D.Williams
B2		"
H		"
R		"
Thermus ruber		"

TABLE 6

Plasmids Used

Plasmids	Genotype, if known	Reference or source
pBR322	Ap^R Tc^R	Bolivar *et. al.*(1977)
pC194	Cm^R	S.D.Ehrlich
pHV33	Cm^R Tc^R Ap^R	"
pCLK1		This work
pCLK2		"
pCLK3		"
pCLK101	Cm^R Ap^R	"
pCLK301	Cm^R	"
pCLK302	Cm^R	"
pCLK303	Cm^R	"

Agarose powder used was SeaKem ME grade from FMC Corp., Marine Colloids Div., Rockland, Maine 04841, obtained through Miles Labs. Ltd., Slough.

Ampicillin was obtained from Beecham Veterinary Products, Crawley, Sussex, as 'Penbritin'.

Antibiotic 'Multodisks' (Code U2 and 30-1 J) were from Oxoid Ltd., Basingstoke, Hampshire.

Bacto-agar, Bacto-tryptone, and Bacto-yeast extract were from Difco Labs., West Molesey, Surrey.

Dialysis tubing - Visking size 1 - 8/32" was from Medicell International Ltd., London.

Millipore nitrocellulose membrane filters (Type GS, 0.22 µm, 25 mm) were from Millipore Corp., Bedford, Mass. 01730, obtained through Millipore (UK) Ltd., London.

Restriction endonucleases (Type II) were bought from Boehringer Mannheim GmbH (through The Boehringer Corp. (London) Ltd., Lewes, East Sussex); BRL Labs., Inc. (through Uniscience Ltd., Cambridge); New England BioLabs., Inc. (through CP Labs. Ltd., Herts), and P-L Biochemicals, Inc. (through International Enzymes Ltd., Windsor, Berks).

T4 DNA ligase was from New England BioLabs, Inc.

Redistilled crystalline phenol, bacterial alkaline phosphatase, and bovine serum albumin were from BRL Labs., Inc.

RNase A was bought from Worthington Biochemical Corp., New Jersey 07728, obtained through Cambrian Chemicals Ltd., Croydon.

λ cI857 S7 DNA was a gift from Dr. M.S. Neuberger, Imperial College. ØX174 RFI DNA was a gift of Mr. M. Slomka, Imperial College.

2.4 Media and buffers

LB medium

This medium was used to culture *B. subtilis* and *E. coli*.

Bacto-tryptone	10 gm
Bacto-yeast extract	5 gm
NaCl	5 gm
Distilled water to	1,000 ml

For solid medium, 15 gm of Bacto-agar were added.

M9 salts

A 10x strength stock solution was prepared, autoclaved,

and stored at room temperature until use. The following components were dissolved successively with stirring at room temperature in 800 ml of distilled water:

Na_2HPO_4	60 gm
KH_2PO_4	30 gm
NaCl	5 gm
NH_4Cl	10 gm
Distilled water to	1,000 ml

M9 medium

This medium was used as the minimal medium for *E. coli*.

M9 salts (x10)	100 ml
Distilled water	887 ml
20% glucose	10 ml
1M $MgSO_4$	1 ml
0.05M $CaCl_2$	2 ml

The components were autoclaved separately and mixed aseptically just before use.

For solid medium, 15 gm of Bacto-agar were added to the distilled water and autoclaved as water agar.

Ramaley and Hixson medium

The growth medium used for culturing *Thermus* strains was

slightly modified from that of Ramaley and Hixson (1970), and contained a higher percentage of tryptone and yeast extract.

Bacto-tryptone	2.0	gm
Bacto-yeast extract	2.0	gm
Castenholz 10x basal salts solution	100	ml
Distilled water to	1,000	ml

The pH of the medium was adjusted to 7.6 at room temperature with 1N NaOH. For solid medium, 25 gm of Bacto-agar were added.

Castenholz 10x basal salts solution

To 960 ml of distilled water, the following chemicals were dissolved successively with stirring at room temperature:

Nitrilotriacetic acid	1.0	gm
$CaSO_4.2H_2O$	0.6	gm
$MgSO_4.7H_2O$	1.0	gm
NaCl	0.08	gm
KNO_3	1.03	gm
$NaNO_3$	6.89	gm
Na_2HPO_4	1.11	gm
$FeCl_3$ solution (0.28 gm/litre)	10	ml
Nitsch's trace element solution	10	ml

The volume was adjusted to 1,000 ml and the pH to 8.2 with 2N NaOH. The stock solution was stored at 4°C unautoclaved.

Nitsch's trace element solution

It contained (per litre of distilled water) :

H_2SO_4 (concentrated)	0.5 ml
$MnSO_4.H_2O$	2.2 gm
$ZnSO_4.7H_2O$	0.5 gm
H_3BO_3	0.5 gm
$CuSO_4.5H_2O$	0.016 gm
$Na_2MoO_4.2H_2O$	0.025 gm
$CoCl_2.6H_2O$	0.046 gm

Medium 162

This medium was designed by Degryse, Glansdorff and Pierard (1978) and was used as a chemically defined minimal medium for the selection and cultivation of prototrophic Thermus strain. It was prepared by mixing three separate components : a 10x basal salts solution, 0.2M $Na_2HPO_4.12H_2O$, and 0.2M KH_2PO_4.

The 10x strength basal salts stock solution was prepared, autoclaved, and stored at room temperature until use. It contained

the following chemicals, dissolved successively with stirring at room temperature in 900 ml of distilled water :

Nitrilotriacetic acid	1	gm
$CaSO_4.2H_2O$	0.4	gm
$MgCl_2.6H_2O$	2	gm
0.01M Ferric citrate	5	ml
Nitsch's trace element solution	5	ml
Distilled water to	1,000	ml

To prepare Medium 162 + Glucose (0.2%) + Ammonium chloride (0.01M), the following components were mixed together :

10x 162 basal salts	100	ml
Distilled water	900	ml
0.2M $Na_2HPO_4.12H_2O$	60	ml
0.2M KH_2PO_4	20	ml
NH_4Cl	0.54	gm

The pH of the medium was adjusted to 7.6 at room temperature with 1N NaOH. After sterilization, 11 ml of 20% glucose solution were added aseptically to the medium.

For solid medium, 25 gm of Bacto-agar were added.

Supplements

When required, L-amino acids were supplemented to a concentration of 20 μg/ml from sterile stock solutions of 5 mg/ml.

Ampicillin was added to a concentration of 100 μg/ml from a stock solution of 20 mg/ml, sterilized by Millipore filtration.

A stock solution of chloramphenicol, 25 mg/ml, was prepared by dissolving 125 mg chloramphenicol powder in 5 ml of ethyl alcohol (96%. v/v). For B. subtilis, chloramphenicol was supplemented to 5 μg/ml, whereas for E. coli, it was supplemented to 20 μg/ml.

Kanamycin sulphate was added to a concentration of 40 μg/ml from a stock solution of 8 mg/ml, sterilized by membrane filtration.

Tetracycline hydrochloride was added to a concentration of 20 μg/ml from a stock solution of 4 mg/ml, which was membrane sterilized.

Buffers

Dilution buffer for serial dilution

Na_2HPO_4	7 gm
KH_2PO_4	3 gm
NaCl	4 gm
$MgSO_4.7H_2O$	0.2 gm
Distilled water to	1,000 ml

Each salt was dissolved in the order given.

TBE buffer

Tris-borate buffer (Peacock and Dingman, 1967) was used for agarose gel electrophoresis. A 10x strength stock solution was prepared, and stored at room temperature until use. It was used as soon as possible because on long storage precipitate appeared in the buffer.

Tris	108 gm
Boric acid	55 gm
$Na_2.EDTA.2H_2O$	9.3 gm
Distilled water to	1,000 ml

The pH of the 1x strength solution was 8.3 .

TE buffer for dissolving DNA and dialysis

10 mM Tris-HCl, pH 7.5

1 mM $Na_2.EDTA$, pH 7.5

2.5 Determination of pH

The pH values of solutions were determined at ambient temperature using a Radiometer pH meter (model pHM 26), fitted with a GK 2302 combined glass electrode and reference cell.

2.6 Sterilization

Media and solutions for use in microbiological work were steam sterilized at 121°C (15 psi) in an autoclave or a Prestige pressure cooker. Volumes up to 400 ml were autoclaved for 20 minutes; whereas larger volumes were autoclaved for longer period. Stock solutions of sugars were normally autoclaved at 110°C (10 psi) for 10 minutes.

Solutions of chemicals which were not stable to heat were sterilized by filtration through sterile membrane filters (Millipore, type GSWP, 0.22 µm pore size, 25mm), positioned in the Millipore Swinnex-25 syringe filter holders.

Glassware, pipettes, polycarbonate centrifuge tubes, Corex glass centrifuge tubes, wooden cocktail toothpicks, velvet pads for replica-plating, Eppendorf 1.5 ml polypropylene microcentrifuge tubes, and Finnpipette disposable polypropylene tips were autoclaved for 60 minutes and dried *in vacuo* for

30 minutes. Millipore Swinnex-25 syringe filter holders together with the Millipore membrane filters were autoclaved for 20 minutes without a drying cycle.

2.7 Maintenance of cultures

For *B. subtilis* and *E. coli*, plates were incubated at 37°C in a dry incubator. Small liquid cultures were grown in sterile 16 x 110 mm screw-capped polystyrene test tubes or sterile screw-capped 30 ml polystyrene universal containers (Sterilin Ltd., Teddington, Middlesex), while larger cultures were grown in Erlenmeyer flasks. They were shaken vigorously in a Gallenkamp orbital incubator (Model INR-200-010V) at 37°C.

For *Thermus* strains, agar plates were incubated in a dry incubator at 58° - 60°C, wrapped in polythene bags to prevent drying out. Normally a beaker filled with tap water was kept in the incubator to keep the atmosphere inside moist, to reduce drying of the plates. The *Thermus* strains were reinoculated onto fresh plates fortnightly.

Small and large liquid cultures of *Thermus* strains were also grown like those mentioned for *B. subtilis* and *E. coli*, except that the temperature was raised to 60°C.

2.8 Plating techniques

i) Determination of viable cell count

The serial dilution method described by Miller (1972) was followed. A dense cell suspension was homogeneously and serially diluted in dilution buffer (Section 2.4) by making a series of dilutions of either 1 : 100 or 1 : 10. Duplicate 0.1 ml samples of suitable dilutions (10^{-6}, 10^{-7}) were spread on appropriate dry plates which were then incubated overnight at the appropriate temperature. Plates having suitable number of colonies (50 - 500) were counted with the aid of a Gallenkamp colony counter.

ii) Replica plating

About 100 colonies were gridded on to a master plate with sterile wooden cocktail toothpicks. After the colonies had grown up, the master plate was pressed lightly onto a sterile velvet square (15 cm x 15 cm), which was stretched firmly over a wooden replicating block of 8 cm diameter by a metal retaining ring. Two to three replicas were made by pressing fresh plates having dry surfaces onto the velvet. The final replica was made either on the same medium as the master plate or on LB plate. Since all colonies should grow on this plate, it

provided a control to check that sufficient cells from each master colony had been transferred onto the velvet.

2.9 Determination of culture scattering

The turbidity of a cell suspension was measured at 650 nm in a Pye-Unicam SP800 spectrophotometer against the appropriate blank.

2.10 Test for antibiotic sensitivity

Plates containing solidified Ramaley and Hixson medium were flooded with a culture of each *Thermus* strain, and air-dried until no free fluid was evident on the agar surface. Oxoid 'Multodisks' (Code U2 and 30-1 J) were then placed on the plates and these were incubated in polythene bags at 58° - 60°C . The inhibition zone size, from the edge of bacterial growth to the edge of the paper disc, was measured after 2 to 4 days.

2.11 Assay for bacteriocin production

The double-layer technique of Fredericq (1957) was used. The plasmid-harbouring *Thermus* strains were stabbed on plates containing solidified Ramaley and Hixson medium and incubated for 48 hours at 58° - 60°C. The macrocolonies which developed

on the plates were killed by exposure to chloroform vapour for 30 minutes, and then overlaid with 5 ml of molten Ramaley and Hixson agar medium containing 0.1 ml of a broth culture of an indicator strain. The indicator strains used were the non-plasmid-bearing Thermus strains, grown overnight in liquid Ramaley and Hixson medium. After overnight incubation at 58° - 60°C, the plates were examined for inhibition zones around the overlaid colonies.

2.12 General techniques for handling DNA

Sterile chromic acid washed glassware was used for the storage and preparation of all nucleic acid samples. Plasmid and chromosomal DNA samples were stored in TE buffer (pH7.5) (Section 2.4) at 4°C in bijou bottles over a few drops of chloroform.

Dialysis tubings were prepared according to the method described by Miller (1972). They were treated sequentially as follows: boiled in 2mM EDTA (pH7.0) and rinsed with distilled water twice, then boiled once in distilled water, 2mM EDTA (pH7.0), rinsed with TE buffer (pH7.5), and finally autoclaved and stored in TE buffer (pH7.5). The sterile tubings were kept at 4°C over a few drops of chloroform. Care was taken to handle them with gloves and not to use any pointed or

sharp objects to stir them.

2.13 Phenol, chloroform-isoamyl alcohol, and ether extraction

A DNA sample in TE buffer was shaken with an equal volume of redistilled phenol which had been equilibrated with TE buffer. The phases were separated by centrifugation and the upper aqueous layer, which contained the DNA, was removed, carefully avoiding the interface layer. The extraction was repeated. After the second phenol extraction, the upper aqueous phase was extracted 2 - 3 times with equal volumes of of chloroform-isoamyl alcohol mixture (24:1, v/v). After the last extraction, the upper aqueous phase was removed into a new tube and the chloroform removed by repeated diethyl ether extractions. The ether was finally blown off by gently bubbling air into the sample, which could then be dialyzed against TE buffer and/or concentrated by ethanol precipitation.

2.14 Ethanol precipitation

To a volume of DNA solution, 1/10 volume of 3M sodium acetate was added, followed by 2 volumes of precooled ethanol (96%, v/v). The mixture was left overnight at -20°C, and the precipitated DNA collected by centrifugation in the Sorvall SS34 rotor at 12,000 rpm at 4°C for 30 minutes. For

volumes less than 1.5 ml, the DNA was precipitated in an Eppendorf microfuge (Model 5412). The DNA pellet was briefly dried in a dessicator and redissolved in the required buffer.

2.15 Determination of DNA concentration

Concentrated DNA samples were normally diluted 20x in TE buffer and their OD_{260} and OD_{280} values determined in a Cecil CE 212 variable wavelength UV monitor. The DNA concentration was deduced from the OD_{260} value, assuming that an absorbance of 1 at 260 nm corresponded to 50 μg/ml of duplex DNA.

The ratio of OD_{260} value to OD_{280} value was used as a criterion of purity. A value of 2 and above was accepted.

2.16 RNase treatment of DNA samples

An aliquot from a stock solution of RNase A (21.35 mg/ml in 0.1% phenol, Worthington Biochemical Corp.) was diluted in 0.15M NaCl, pH5.0, and heated at 80°C for 10 minutes to inactivate any contaminating DNase (Marmur, 1961). After cooling, the heat treated RNase was added to the nucleic acid solution to a final concentration of 25 μg/ml, and the mixture was incubated at 37°C for 30 minutes with gentle shaking.

2.17 Isolation of plasmid DNA from *E. coli*

Basically, the dye-buoyant density centrifugation method described by Clewell and Helinski (1969) and Clewell (1972) was followed.

A single colony of *E. coli* grown on LB plate supplemented with the appropriate antibiotic(s) was inoculated into 10 ml of LB broth plus antibiotic(s), which was grown overnight at 37°C. The overnight culture was used to inoculate 200 ml of LB broth plus antibiotic(s) in a 500 ml Erlenmeyer flask (1 : 100 dilution), which was then incubated to saturation overnight at 37°C in a Gallenkamp orbital shaker at 200 rpm. For cultures containing 'ColE1' type plasmid (e.g. pBR322), at OD_{600} of approximately 0.6, chloramphenicol was added to a concentration of 150 μg/ml and the flask continued shaking at 37°C overnight (about 15 - 20 hours).

Cells were harvested by centrifugation, washed with 100 ml of cold TE buffer (pH7.5), and resuspended at 0°C in 2.25 ml of iced 25% sucrose in 0.05M Tris-HCl, pH8.0 . Lysis was achieved by adding 0.75 ml of a freshly prepared lysozyme solution (10 mg/ml in water). After the suspension was maintained for 5 minutes on ice, with occasional gentle swirling, 0.75 ml of iced 0.5M EDTA (pH8.0) was added. The mixture was

again swirled gently on ice for 5 minutes. Finally, 3.75 ml of iced Triton lysis buffer (containing 1 ml 10% Triton X-100, 12.5 ml 0.5M EDTA, pH8.0, 5 ml 1M Tris-HCl, pH8.0, and 80 ml distilled water) were added to the mixture. The lysate, which became relatively clear and viscous, was swirled gently on ice for another 10 - 15 minutes, and then cleared by centrifugation at 20,000 rpm for 30 minutes at 4°C, using a Sorvall SS34 fixed angle rotor.

The cleared lysate was collected into a nitrocellulose centrifuge tube, and its volume measured. One gram of CsCl and 0.1 ml of 5 mg/ml ethidium bromide solution were added for each millilitre of lysate, and the lysates were centrifuged at 38,000 rpm for 60 hours at 18°C in a Beckman 50Ti fixed angle rotor.

At the end of the spin, the DNA bands were visualized by illuminating the tube with long-wavelength UV light, and the lower band which contained the denser ccc plasmid DNA was collected by puncturing the side of the tube with a 19-G hypodermic needle. Ethidium bromide was removed by extracting three to four times with isopropanol that had been equilibrated with water and solid CsCl. The resulting colourless ccc plasmid DNA solution was then dialyzed against 2 to 3

litres (two changes) of TE buffer, and stored at 4°C until use. The OD_{260} and OD_{280} values of the plasmid DNA solution were measured ; and so was its volume.

2.18 Rapid extraction of plasmid DNA from *E. coli*

Initially, the method of Birnboim and Doly (1979) was used. Later, the procedure of Kado and Liu (1981) was preferred because it was less time-consuming.

Birnboim and Doly's method (1979)

0.5 ml of an overnight culture in LB medium at 37°C was transferred to a 1.5 ml Eppendorf tube, and the cells pelleted by a 30 second centrifugation in an Eppendorf microfuge (Model 5412). The supernatant was carefully removed with a fine-tip Pasteur pipette and the cell pellet was thoroughly suspended in 100 µl of freshly prepared lysis solution (2 mg/ml lysozyme, 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH8.0). After a 30 minute period of incubation at 0°C, with occasional mixing, 200 µl of alkaline-SDS solution (0.2N NaOH, 1% SDS) were added and the tube was gently vortexed. The suspension became almost clear and slightly viscous. The tube was maintained for 5 minutes at 0°C, and then 150 µl of 3M sodium acetate (pH4.8) were added. After gently mixing the content of the tube by inversion for a few seconds, the tube was

left at 0°C for 60 minutes with occasional mixing to allow precipitation of high molecular weight RNA, protein-SDS-complexes, and chromosomal DNA, which formed an aggregate of insoluble network.

The tube was then centrifuged for 5 minutes to sediment the precipitate and to yield an almost clear supernatant. 400 µl of the supernatant were transferred to a second Eppendorf microcentrifuge tube, avoiding any of the floating materials. One ml of cold ethanol (96%, v/v) was added and the tube was held at -20°C for 30 minutes. The precipitate was collected by centrifugation for 2 minutes, and the supernatant removed by aspiration. The pellet was dissolved in 100 µl of 0.1M sodium acetate (pH6.0) and reprecipitated with 200 µl of cold ethanol (96%, v/v). After 10 minutes at -20°C, the precipitate was again collected by centrifugation for 2 minutes. The pellet was dissolved in 75 µl of TE buffer.

The resulting DNA solution was used for agarose gel electrophoresis (applying 25 µl to the gel), transformation, and endonuclease digestion (after treating it with RNase).

<u>Kado and Liu's method (1981)</u>

0.5 ml of an overnight culture in LB medium at 37°C was

transferred to a 1.5 ml Eppendorf microcentrifuge tube and the cells pelleted by a 30 second centrifugation in an Eppendorf microfuge (Model 5412). The supernatant was carefully removed with a fine-tip Pasteur pipette and the cell pellet was thoroughly suspended in 100 µl of lysing solution (3% SDS in 50 mM Tris, pH12.6). The lysate was incubated at 60°C for 60 minutes, with occasional mixing, and then briefly emulsified with an equal volume of distilled phenol-chloroform mixture (1 : 1, v/v). The mixture was separated into three layers by centrifugation for 5 minutes in a Beckman horizontal microfuge (Model B). The upper aqueous phase was used directly for agarose gel electrophoresis to screen for plasmid or hybrid plasmid DNA molecules. Usually, 20 µl were withdrawn and mixed with 4 µl of bromophenol blue dye (Section 2.23) on a square of parafilm M (American Can Company, Greenwich, Conn.).

For transformation and endonuclease digestion, the upper aqueous phase was carefully transferred into a new Eppendorf microtube, avoiding the precipitate at the interface, and then freed of phenol by repeated extractions (three to four times) with water-saturated diethyl ether. The residual ether was blown off with air before the DNA was precipitated by adding one-tenth volume of 3 M sodium acetate and two volumes of

cold ethanol (96%, v/v). The mixture was incubated overnight at -20°C or in a dry ice-isopropanol bath for 5 minutes. The precipitated DNA was pelleted by centrifugation for 10 minutes, dried briefly in vacuo, and suspended in 100 µl of TE buffer. 20 µl of the solution were sufficient for restriction endonuclease digestions and transformation.

2.19 Isolation of plasmid DNA from B. subtilis

This was performed by the method described by Niaudet and Ehrlich (1979).

Cells were collected from 200 ml of an overnight culture in LB medium and the selective antibiotic, washed with 100 ml of a buffer containing 0.1M NaCl, 0.05M Tris-HCl, pH7.5, and 1mM EDTA, pH7.5, and resuspended in 10 ml of 25% sucrose, 0.1M NaCl, and 0.05M Tris-HCl, pH7.5. Fresh lysozyme solution (0.75 ml, 20 mg/ml in water) was added and the mixture incubated at 37°C for 20 - 30 minutes in a reciprocal shaking water bath. The following solutions were then added in order: 2.4 ml of 5M NaCl, 0.6 ml of 0.5M EDTA, pH8.0, and 12.5 ml of freshly made 2% SDS - 0.7M NaCl. The suspension was gently but thoroughly mixed and left overnight at 4°C. The following day, the lysate was centrifuged for 30 minutes at 18,000 rpm at 4°C in a Sorvall SS34 rotor, and the super-

natant ('cleared lysate') was collected and adjusted to 1M NaCl by adding 5M NaCl. One-third volume of 40% PEG 6000 was then added to precipitate the DNA, and the suspension incubated for 2 hours at 0°C. The precipitate was collected by centrifugation and dissolved in 5 ml of TE buffer (pH7.5).

Plasmid DNA was then isolated by CsCl-EtBr density gradient centrifugation as described in Section 2.17 for _E. coli_ and similarly treated after the centrifugation.

2.20 Preparation of total DNA from _Thermus_

The procedure used was a modification of that described by Marmur (1961).

An overnight culture of _Thermus_, grown in 200 ml of Ramaley and Hixson liquid medium at 60°C, was pelleted by centrifugation and resuspended in 50 ml of 0.15 M NaCl - 0.1 M EDTA, pH8.0. The cell suspension was then frozen at -20°C. After thawing at room temperature, the cells were harvested by centrifugation and resuspended in 6 ml of 0.15M NaCl - 0.1M EDTA. The homogeneous cell suspension was transferred into a 100-ml Erlenmeyer flask to ensure easy mixing of the flask's content in the subsequent steps. 10 mg of lysozyme were added to the cell suspension and the mixture was incubated at

37°C in a reciprocal shaking water bath for 30 minutes with gentle shaking. Lysis of the cells was observed and there was a marked increase in viscosity. Complete lysis was effected by the addition of 0.75 ml 25% SDS (w/v). The lysate was mixed gently and then placed in a 60°C water bath for 10 minutes. It was cooled to room temperature before the addition of 2 ml 5M sodium perchlorate solution.

To deproteinize the lysate, an equal volume of chloroform-isoamyl alcohol (24 : 1, v/v) was added to the viscous, lysed suspension and the whole mixture was shaken for 20 minutes in the Erlenmeyer flask, the mouth of which was covered with a piece of cling film, with the aid of a wrist-action flask shaker. The resulting white emulsion was poured into a sterile acid-washed 30-ml Corex centrifuge tube, and was separated into three layers by an 8 minute centrifugation at 8,000 rpm in a Sorvall SS34 rotor at ambient temperature. The upper aqueous phase containing the nucleic acids was carefully pipetted off, without disturbing the interface layer at which the denatured protein accumulated, with a wide-mouth Pasteur pipette into a clean 100-ml Erlenmeyer flask. The chloroform deproteinization step was repeated once.

After the final deproteinization, the upper aqueous phase was carefully pipetted into a 30-ml Corex centrifuge tube,

and its volume measured. One-tenth volume of 5M potassium acetate was added to the aqueous phase to precipitate the remaining cell debris, proteins, and SDS. The mixture was incubated overnight at 0°C. On the following day, the white precipitate containing cell debris and potassium-SDS-protein complex was sedimented by a long centrifugation in the Sorvall SS34 rotor at 0°C. The supernatant was gently decanted into a large boiling tube, and its volume measured. Two volumes of cold ethyl alcohol (96%, v/v) were then gently layered on the supernatant by adding it slowly down the inner wall of the tube. The nucleic acids were precipitated at the interface upon gentle rotation of the tube and were collected by winding them on a glass rod as a fibrous threadlike precipitate. The spooled DNA fibres were washed once with alcohol, drained free of excess alcohol by pressing the spooled rod against the inner wall of the tube, and air-dried before being redissolved in 5 ml of TE buffer.

2.21 Acid-phenol extraction

The method described by Zasloff, Ginder and Felsenfeld (1978) was used.

1/20 volume of 1M sodium acetate, pH4.0, and 1/20 volume of 1.5M NaCl were added to a volume of DNA solution, and was

followed by one volume of redistilled phenol, equilibrated with 50mM sodium acetate, pH4.0. The mixture was vortexed for 2 - 3 minutes. The resulting white emulsion was centrifuged in a Beckman horizontal microfuge (Model B) for 5 minutes to separate into three phases. The upper aqueous phase, which contained the ccc plasmid DNA, was extracted with acid-phenol twice. After the final acid-phenol extraction, 1/20 volume of 1M Tris-HCl, pH8.6, was added to neutralize the aqueous phase, which was then extracted with ether for four to five times to remove the phenol. Finally, the ether was blown off by bubbling air gently into the sample.

2.22 Isolation of plasmid DNA from *T. aquaticus*

The method used was adapted from Guerry *et al.* (1973), Humphreys *et al.* (1975) and Schwinghamer (1980).

T. aquaticus cells were grown at 60°C in 200 ml of Ramaley and Hixson medium supplemented with 0.15% (w/v) ammonium acetate. An overnight culture was harvested and resuspended in 100 ml of TE buffer, pH7.5. 0.5 ml of 20% SDS (w/v) was added to the cell suspension which was then immediately vortexed for 30 - 60 seconds. The cells were collected by centrifugation in a Sorvall GSA rotor at 8,000 rpm for 8 minutes. After decanting the supernatant, the cells were thoroughly

resuspended in 10 ml of 25% sucrose, 0.1M NaCl, and 0.05M Tris-HCl, pH7.5, by pipetting the cell suspension up and down several times with a 10 ml pipette. The homogeneous cell suspension was transferred into a 125-ml Erlenmeyer flask to facilitate mixing in the subsequent steps. Fresh lysozyme solution (0.75 ml, 20 mg/ml in water) was added and the mixture incubated at 42°C for 5 minutes, and then at 37°C for another 30 minutes with gentle shaking in a reciprocal shaking water bath. The following solutions were then added in order : 2.4ml of 5M NaCl, 0.6ml of 0.5M EDTA, pH8.0, and 12.5ml of freshly prepared 2% SDS-0.7M NaCl. The suspension was gently but thoroughly mixed and left overnight at 4°C. The following day, the lysate was centrifuged for 30 minutes at 18,000 rpm at 4°C in a Sorvall SS34 rotor and the supernatant ('cleared lysate') was collected.

Supernatants from two 200 ml cultures were pooled and the total volume measured in a sterile graduated measuring cylinder. It was adjusted to 1M NaCl by adding 5M NaCl. One-third volume of 40% PEG 6000 was then added to precipitate the DNA and the suspension incubated for 2 - 3 hours at 0°C. The precipitate was collected by centrifugation in the Sorvall GSA rotor at 7,000 rpm for 8 minutes at 0°C, and was

dissolved in 7.5 ml of TE buffer, pH7.5. The volume of the DNA solution was adjusted to 8 ml with TE buffer, and 7.5 gm CsCl and 0.28 ml of EtBr solution (10 mg/ml) were added.

Plasmid DNA was then isolated by CsCl-EtBr density gradient equilibrium centrifugation as described in Section 2.17 for *E. coli* and treated similarly after the centrifugation.

2.23 Agarose gel electrophoresis

Total cellular DNA, plasmid DNA, and restriction endonuclease generated DNA fragments were resolved by electrophoresis in submerged horizontal 0.4% to 2.0% agarose slab gels in Tris-borate buffer, pH8.3 (Peacock and Dingman, 1967) (Section 2.4).

The electrophoresis was performed in a BRL Horizontal Gel Electrophoresis System (Model H2, 14 cm long x 11 cm wide) or a mini-gel system (5.5 cm long x 8 cm wide) designed in the course of this study.

To prepare agarose gels, a weighed amount of agarose powder (SeaKem ME grade) in the Tris-borate buffer was melted by boiling it in a pressure cooker for 3 - 5 minutes at 5 psi. The homogeneous molten agarose solution was cooled to around

$50^{o}C$ before pouring onto the perspex tray, and allowed to set around an appropriately sized perspex comb, the teeth of which formed the sample wells. The sample-comb was adjusted before pouring the gel so that the bottoms of the sample-comb teeth were kept about 0.5 mm above the gel bed. In this way, the sample wells did not go all the way through the gel.

After the gel had completely set, the comb was removed with a gentle lifting motion and the wells were filled with electrophoresis buffer, being careful to remove any air bubbles from the wells. The gel was then laid horizontally in the buffer tank of the electrophoresis system and was completely submerged by about 1 mm in the electrophoresis buffer, i.e. Tris-borate buffer.

DNA samples containing 10% sucrose, 0.01% bromophenol blue, 5mM EDTA, 2.5mM Tris-HCl, pH7.5, and 0.25% ficoll were carefully loaded beneath the buffer in the appropriate wells with a drawn out glass micropipette. Care was taken not to overfill the wells with DNA samples.

Electrophoresis was carried out at room temperature from cathode (-) to anode (+), and voltage gradients normally employed were between 0.5 and 5 V/cm . For high-molecular-weight DNA fragments (1.5 kb and above) , better resolution

was achieved at low-voltage gradient in a low percentage gel, e.g. 0.4% - 0.7% . Conversely, low-molecular-weight DNA fragments (1 kb and below) which diffused through the gel were best separated at fairly high-voltage gradients in a high percentage gel, e.g. 1.8% - 2.0%.

At the end of the run, the DNA was stained by soaking the gel in 5 µg/ml of EtBr solution for about 30 minutes. After destaining the gel in water for another 15 - 30 minutes, the DNA molecules complexed with EtBr were visualized and photographed on a 254-nm UV transilluminator (C61, UV Products, Inc.), using a number 15 Kodak Wratten gelatin yellow filter and a MP-4 Land Camera (Polaroid) loaded with Polaroid Type 665 black-and -white Land films. The exposure times varied from 3 - 8 minutes.

Analytical gels were performed in the BRL H2 system, which required 50 ml - 100 ml of molten agarose; whereas, the mini-gel system provided a convenient, rapid means to screen for plasmids, and to monitor the activities of restriction endonucleases and ligases. To prepare a mini-gel, 15 ml - 20 ml of molten agarose were required, and EtBr was added to the molten agarose at 0.5 µg/ml just before pouring the gel to avoid having to stain and destain it after electrophoresis. Normally, it took 60 - 90 minutes to run a mini-gel

TABLE 7

Restriction Endonucleases Used

Enzyme	Source	Assay buffer
AluI	BRL	M
AvaII	BRL	M
BclI	BRL	M
BglI	BRL	M
BglII	BM	L
BstI	CC	M
BstEII	BRL	M
EcoRI	BM	H
EcoRII	BRL	M
HaeII	BRL	L
HaeIII	MSN	L
HindIII	BM	M
HinfI	MSN	M
HpaI	BRL	L
HpaII	BM	L
KpnI	BRL	L
MboI	PLB	H
MboII	PLB	L
PstI	BM	M
SalI	NEB	H
SmaI	BRL	TMK
SstI	BRL	H
SstII	BRL	H
TaqI	BM	L
XbaI	BRL	H
XorII	BRL	L

L : Low salts assay buffer
M : Medium salts assay buffer
H : High salts assay buffer

	NaCl	Tris-HCl pH 7.5	$MgSO_4$	DTT
L :	-	10mM	10mM	1mM
M :	50 mM	10mM	10mM	1mM
H :	100mM	50mM	10mM	-

TMK : 15mM Tris-HCl, pH8.0
6mM $MgCl_2$
15mM KCl

BM : Boehringer Mannheim
BRL : Bethesda Research Laboratories, Inc.
CC : Dr. Catherine Clarke
MSN : Dr. Michael S. Neuberger
NEB : New England BioLabs, Inc.
PLB : P-L Biochemicals, Inc.

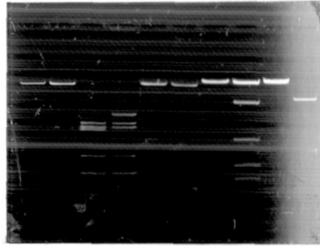
with a voltage gradient of 5 - 8 V/cm. Samples of the results electrophoresed in the mini-gel system are shown in Fig. 7 (i) and (ii).

2.24 Digestion of DNA with restriction endonucleases

The restriction endonucleases used in this work together with their sources and the composition of their assay buffers are listed in Table 7. Four basic assay buffers (Low salts, Medium salts, High salts, and TMK) were used, and they were prepared as 10x strength stock solutions.

Digestions were carried out in sterile 1.5 ml Eppendorf polypropylene microtubes, in a final reaction volume of 10 - 50 μl, containing 0.1 - 1.0 μg of DNA and 0.5 - 2 units of restriction endonuclease. The reaction mixtures were mixed well and incubated for 60 - 120 minutes at 37°C, except for *BclI* (50°C), *BstEII* (60°C), and *TaqI* (60°C). At the end of the incubation, reactions were terminated by heating at 70°C for 5 minutes to inactivate the restriction endonucleases and to melt cohesive ends, before being applied to a gel for electrophoresis.

For double endonuclease digestions, enzymes which required assay buffers of the same NaCl concentration were used to

(i) 

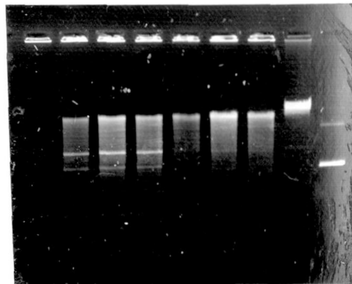
(ii) 

Fig. 7 (i) and (ii). Samples of agarose gel electrophoresis performed in the mini-gel system.

(i) An agarose gel (1.0%) monitoring the activities of restriction endonucleases on plasmids .

(ii) An agarose gel (0.5%) monitoring the activities of T4 DNA ligase.

digest the DNA simultaneously in the common assay buffer. In other cases when the salt concentrations were different, digestions were carried out sequentially with the two enzymes. After incubation with the first enzyme, which required lower salt concentration, the sample was heated for 5 minutes at 70°C, and buffer components were added to bring the composition of the buffer as close as possible to that desired for the second enzyme, before it was added to the digestion mixture.

2.25 Determination of molecular weights of DNA fragments in agarose gel

The graphical method of relating the logarithm of the molecular weight of a DNA molecule (logM) to its electrophoretic mobility (m) in gels was used to determine the molecular weights of restriction endonuclease generated DNA fragments (Aaij and Borst, 1972; Sharp, Sugden and Sambrook, 1973; McDonell, Simon and Studier, 1977; Southern, 1979).

Restriction fragments of known molecular weights produced from the DNA molecules of λcI857 S7, ØX174 RFI, and pBR322 (Table 8) were used as standards for calibrating the sizes of linear DNA fragments.

TABLE 8

SIZES OF RESTRICTION FRAGMENTS OF λ DNA, ØX174 RF DNA & pBR322

λ EcoRI BstI	λ HindIII	ØX174 PstI	ØX174 HpaI	ØX174 HpaII	ØX174 HaeII	ØX174 HaeIII	pBR322 AluI	pBR322 HinfI
16.17	23.72	5.39	3.73	2.75	2.31	1.35	0.910	1.631
(8.87)@	9.46		1.26	1.70	1.57	1.08	0.659	0.517
5.59	6.67		0.392	0.374	0.783	0.872	0.655	0.506
4.7	4.26			0.348	0.269	0.603	0.521	0.396
(x2)3.77	2.25			0.219	0.185	0.310	0.403	0.344
3.53	1.96				0.123	0.281	0.281	0.298
3.28	0.59				0.093	0.271	0.257	0.221
2.79	0.10				0.054	0.234	0.226	0.220
2.55						0.194	0.136	0.154
1.81						0.118	0.100	0.075
1.13						0.072	0.063	
							0.057	
							0.049	
							0.019	
							0.015	
							0.011	

NOTE :

All values are in kilobase-pairs (kb).

The full size of λDNA is taken as 49 kb (Phillipsen, Kramer and Davis, 1978). The values of λ-EcoRI and BstI are from Thomas and Davis (1975) and Haggerty and Schleif (1976). The values of λ-HindIII are from Phillipsen et al. (1978).

@ Created by the joining of the 3.28kb and 5.59kb fragments through the λ cohesive end sequences.

The values of ØX174 are from Sanger et al. (1978).

The values of pBR322 are from Sutcliffe (1978).

EcoRI cleaves the 1.631 kb fragment of pBR322-HinfI into 2 fragments of 0.996 kb and 0.635 kb.

The distance travelled from the well for each DNA fragment (m) was measured from the negatives. A standard curve was obtained by plotting the log molecular weight of the standard DNA fragments (logM) versus their mobilities (m) (two examples are given in Fig. 7 (iii) and (iv), one involving the larger fragments produced from λ and ØX174, and the other the smaller fragments from pBR322). From the standard graph, the molecular weights of linear DNA fragments of unknown size were determined. The avarage of 2 - 4 estimates was used.

2.26 Ligation of DNA fragments

In general, equal amounts of digested vector DNA and insert DNA were mixed in a reaction mixture containing 50 mM Tris-HCl (pH7.8), 10 mM $MgCl_2$, 20 mM DTT, 1.0 mM ATP, and 50 μg/ml BSA, to give a final DNA concentration of 20 μg/ml. 0.5 - 1 Weiss unit of T4 DNA ligase (New England BioLabs, Inc.) was added to the mixture, which was then incubated at 15°C for about 12 - 18 hours. The extent of ligation was monitored by gel electrophoresis. Before the addition of the ligase, a sample of the mixed DNA was always removed, and was used to run alongside of the ligated sample to compare the extent of ligation.

Fig. 7 (iii). Plot of electrophoretic mobility (m) vs log of M.W. of the restriction DNA fragments of λ and ØX174.

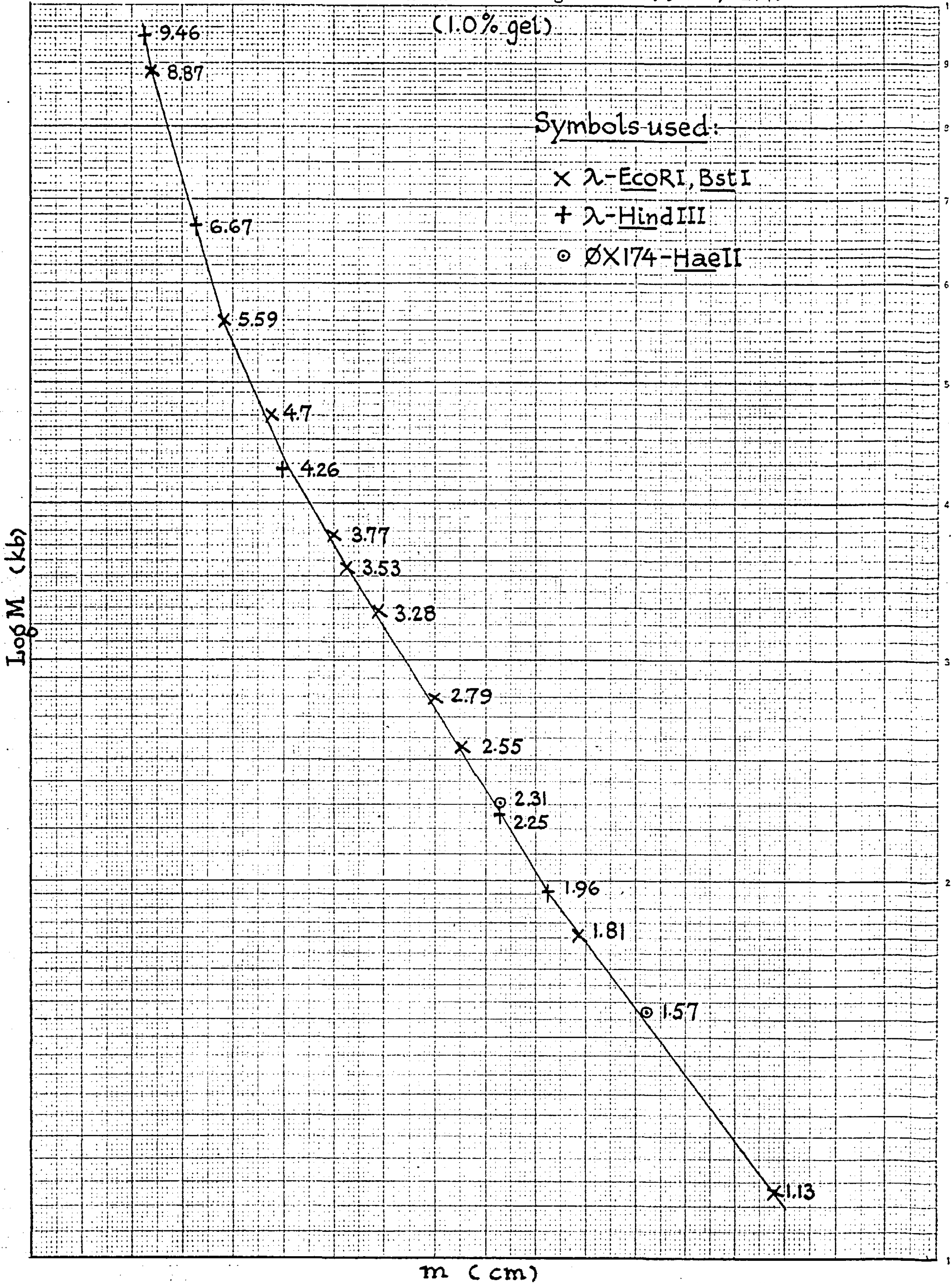
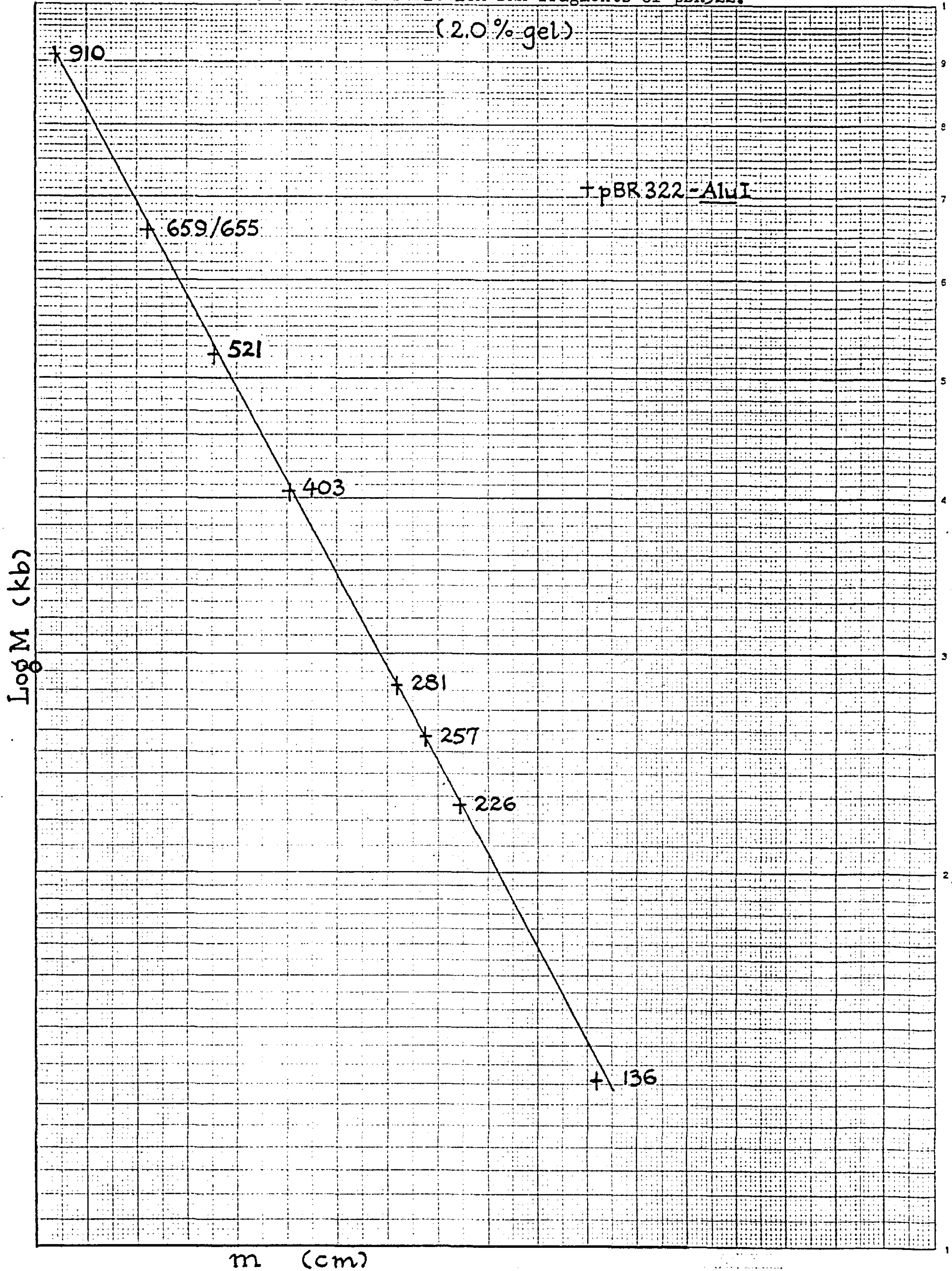


Fig. 7 (iv). Plot of electrophoretic mobility (m) vs log of M.W. of the restriction DNA fragments of pBR322.



Sometimes, prior to ligation, the endonuclease-digested vector DNA having sticky termini was treated with bacterial alkaline phosphatase (28 units per μg of DNA, BRL, Inc.) at 65°C in 10 mM Tris-HCl (pH8.0) for 30 minutes, followed by phenol extraction, to prevent self-ligation, intramolecularly as well as intermolecularly (Ullrich *et al.*, 1977).

2.27 Transformation of *E. coli* with plasmid DNA

Initially, the procedure used was based on that of Mandel and Higa (1970), as described by Glover (1976). Later, the method of Dagert and Ehrlich (1979) was used.

An overnight culture of *E. coli* in LB broth, grown up from a single colony, was used to inoculate 100 ml of LB liquid medium (1 : 100 dilution), which was then incubated, with shaking at 200 rpm, at 37°C until the OD_{650} reached 0.2. The culture was immediately chilled for 10 minutes on ice. The cells were pelleted by centrifugation and resuspended in 40 ml of cold (0° - 4°C) 0.1M $CaCl_2$ and held at 0°C for 20 - 25 minutes. The cells were again harvested by centrifugation and resuspended in 1 ml of 0.1M $CaCl_2$. They were then kept in ice for 24 hours before being used for transformation.

Transformation was performed by mixing 0.1 ml of chilled, $CaCl_2$ - treated E. coli cell suspension with 0.02 ml of suitably diluted plasmid DNA solution or ligated DNA solution. The mixture was incubated on ice for 10 minutes, and then at 37°C for 5 minutes, before being diluted with 2 ml of LB medium and incubated at 37°C with shaking for 60 minutes to allow phenotypic expression of genetic determinants on the plasmid DNA. The cells were pelleted and resuspended in a suitable volume of growth medium to spread on selective plates. Samples were also plated on nonselective plates to estimate the viable cell count and the frequency of transformation.

CHAPTER THREE

SCREENING OF *THERMUS* SPECIES FOR CCC PLASMID DNA

3.1 Introduction

At the outset of this research project, it was decided that a plasmid replicon was preferred to a bacteriophage replicon as the cloning vector in *Thermus*. It was also decided to perform some exploratory experiments to test whether any of the existing plasmids could be used as cloning vectors in *Thermus*, which is classified in the same group, the 'Gram-negative aerobic rods and cocci' group, as *Pseudomonas* and *Rhizobium* in *Bergey's Manual of Determinative Bacteriology*, 8th edition (1974). The plasmid RP4, belonging to the promiscuous incompatibility group P, was used as it was shown to have a wide host range and had been successfully transferred to many Gram-negative bacterial species, including *Rhizobium* and *Pseudomonas*, from which it was originally isolated (Datta and Hedges, 1972; Thomas, 1981). Attempts were made to transfer RP4 into *T. aquaticus* via conjugation and transformation, using the procedures established in *E. coli* and *Pseudomonas*. However, these preliminary attempts were not successful.

Many reasons could account for the failure to

isolate T. aquaticus strains exhibiting the phenotypic traits of RP4. It could be that the conjugation or transformation procedures used were not effective for Thermus. It might be due to the inability of RP4 to replicate or express in Thermus, or it might be possible that the T. aquaticus strains used were not suitably receptive to exogenous DNA molecules.

To eliminate all these probabilities, it was reasoned that the realistic approach to this problem would be to look for plasmids originating in Thermus, characterize them, and then later use these thermophilic replicons to develop a transformation procedure in Thermus.

A prerequisite to using plasmid DNA as a cloning vector in a cloning system is the ability to isolate and detect the plasmid DNA molecules in the host bacterium with ease. To this end, as mentioned in Chapter One (Section 1.3), many rapid procedures for isolating and detecting plasmids had been developed over the last few years in a wide range of bacterial species. All these methods were based on the fact that plasmids exist in the cytoplasm of cells as supercoiled ccc DNA molecules and this configuration facilitates their isolation from the bacterial cells. These methods generally relied first upon the resistance of plasmid DNA to extraction conditions which disrupted the bacterial chromosome, and

secondly upon the ease with which the plasmid ccc DNA was separated from the chromosome debris by agarose gel electrophoresis for detection. It would be advantageous and convenient to have a similar method developed for Thermus species.

3.2 Method and results

At the beginning of this project, no plasmid had been reported in Thermus, and most of the methods available then were for isolating and detecting plasmids in E. coli. The 'cleared lysate' method of Clewell and Helinski (1969) was tried on a number of Thermus strains to screen for the presence of ccc DNA molecules in them, but no satellite band was detected after the CsCl-EtBr equilibrium density centrifugation.

However, in the process of isolating total cellular DNA from the Thermus strains using a modified procedure of Marmur (1961) (Chapter Two, Section 2.20), additional DNA bands were observed in some strains when the DNA samples were electrophoresed in an agarose gel. T. aquaticus strains B1, B2 and H appeared to contain extra DNA bands, in addition to the main chromosomal DNA band (Fig. 8). These bands were indeed DNA molecules because they remained there after

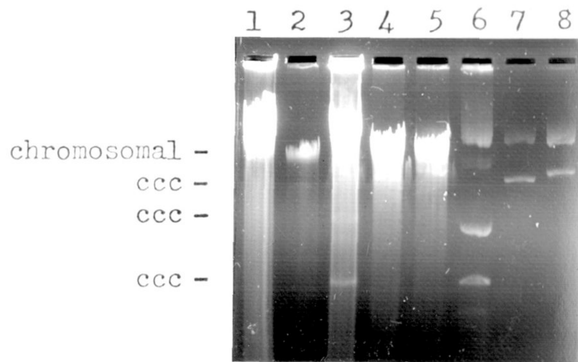


Fig. 8. Agarose gel electrophoresis (0.7%) of DNA iso- from _Thermus_ species. Total DNA extracted from (1) _T. ruber_ ; (2) _T. aquaticus_ R ; (3) _T. aquaticus_ H ; (4) _T. aquaticus_ B1 ; and (5) _T. aquaticus_ B2.

DNA from the water phase after acid-phenol extraction of (6) _T. aquaticus_ H ; (7) _T. aquaticus_ B1 ; & (8) _T. aquaticus_ B2.

the DNA samples had been treated with RNase.

Later, these bands were shown to be extrachromosomal ccc molecules and not just some linear chromosomal fragments by the acid-phenol extraction method of Zasloff *et al.* (1978) (Chapter Two, Section 2.21). When samples of *T. aquaticus* B1, B2 and H total cellular DNA solutions were extracted with acid phenol, the bulk of the linear chromosomal DNA was extracted into the acid-phenol phase, leaving the ccc DNA molecules in the aqueous phase after the extraction. Consequently, the main chromosomal band disappeared from the gel when DNA from the aqueous phase was applied to an agarose gel electrophoresis. The extrachromosomal ccc DNA bands still remained visible in the gel (Fig. 8).

3.3 Discussion

A simple and reliable method had been developed to screen for the presence of extrachromosomal ccc DNA molecules in *T. aquaticus*. Among the few *Thermus* strains tested, *T. aquaticus* B1, B2 and H were found to possess plasmid DNA molecules. This is the first evidence for the presence of ccc plasmids in *T. aquaticus* strains.

This isolation procedure, which was adapted from that of

Marmur (1961), was in fact commonly used in the 1960's during the early studies on bacterial plasmids (Roth and Helinski, 1967; Clowes, 1972). At that time, however, the acid-phenol extraction method of Zasloff et al. (1978) and agarose gel electrophoresis were not available, and detection of the ccc molecules was based on the more laborious and time-consuming methods of sucrose gradient sedimentation, density gradient equilibrium centrifugation, and electron microscopy.

This screening procedure is much superior to the generally used methods which depend on isotopic labelling of the bacterial DNA followed by either CsCl-dye buoyant isopynic density gradient centrifugation or rate zonal centrifugation, and measurement of the distribution of isotope within the gradient. These methods are extremely tedious and time-consuming, especially if they re used just for screening bacterial strains for the presence of ccc plasmid DNA.

During the course of this study, more rapid extraction procedures became available, especially for E. coli. The methods of Birnboim and Doly (1979) (Chapter Two, Section 2.18) and Kado and Liu (1981) (Chapter Two, Section 2.18) were tried on T. aquaticus B1, B2 and H. Although plasmid

DNA in E. coli could be clearly detected by these methods, the plasmid DNA in T. aquaticus strains could not be consistently detected by them. The difficulty in detecting the plasmid DNA in Thermus seems to be due to impurities different from those present in E. coli. The large amount of bright yellow pigments and slime from Thermus (Williams, 1975; Pask-Hughes and Williams, 1977) may interfere with agarose gel electrophoresis and DNA extraction. Denatured chromosomal DNA debris from these strains may not be completely removed, presumably because of differences in properties between the chromosomal DNA of E. coli and Thermus. These two methods need to be modified and improved in order to become successful rapid plasmid extraction procedures in Thermus.

In summary, a method was developed for the screening of plasmid DNA in T. aquaticus. Evidence was provided to show that three of the strains do harbour plasmid DNA molecules.

CHAPTER FOUR

ISOLATION OF PLASMID DNA FROM *THERMUS*

4.1 Introduction

Having detected ccc plasmid DNA molecules in some *T. aquaticus* strains, the next step necessary was to isolate them so that they could be characterised. Many methods had been developed to isolate and prepare large amounts of pure plasmid DNA molecules from a variety of bacterial cells. As mentioned in Chapter One (Section 1.3), these methods were based upon the supercoiled ccc configuration of plasmid DNA molecules within the bacterial cells and the unique properties associated with it. Generally, the plasmid-harbouring cells were first gently lysed to yield a 'cleared lysate' which was then either centrifuged directly in CsCl containing a saturating amount of EtBr or treated to enrich the plasmid molecules prior to isopynic centrifugation in a CsCl-EtBr gradient.

As there was no known method for the isolation of plasmid DNA molecules from *Thermus* species, it was essential to develop one such procedure.

4.2 Method and results

Initially, the 'cleared lysate' method described by

Clewell and Helinski (1969) was used to isolate the *Thermus* plasmids. However, no satellite plasmid band was detected after the CsCl-EtBr equilibrium density centrifugation. This is not an uncommon problem as it is generally known that plasmid isolation methods which have been used and described for a particular bacterial species, especially *E. coli*, need not necessarily be as successful in another bacterial species. The main problem lies in the production of a satisfactory cleared lysate, and with organisms other than *E. coli* and *B. subtilis*, it is frequently a combination of skill, luck and patience to produce satisfactory cleared lysates (Old and Primrose, 1980).

The problem in this case, as in many other situations, was thought to be very likely due to inadequate lysis of the *Thermus* cells, which are yellow-pigmented and covered with an extracellular slime layer (Williams, 1975). This layer would serve as an effective barrier to prevent the entry of lysozyme to attack the cell wall. An improved lysis method had therefore to be devised. After a survey of the various techniques described in the literature, a reproducible method (outlined in detail in Chapter Two, Section 2.22) that combined the procedures of Radloff *et al.* (1967), Guerry *et al.* (1973), Humphreys *et al.* (1975), and Schwinghamer (1980) was developed to isolate the ccc plasmid DNA molecules from *T. aquaticus* strains.

To improve lysis, before the addition of freshly prepared lysozyme solution, the *Thermus* cells were first washed in dilute detergent (0.1% SDS, v/v). This detergent wash (Schwinghamer, 1980) predisposed the cell surface to lysozyme penetration. Immediately after this treatment, the cells were harvested and resuspended thoroughly in 25% sucrose solution. At this stage, it was important to have a homogeneous cell suspension free of any lumps, and this was achieved by pipetting the cell suspension up and down several times with a 10 ml pipette. The cells were lysed by the combined action of lysozyme and SDS. Incubation of the cells with lysozyme at a higher temperature of 42°C & 37°C improved the lysis of the sensitized cells. Complete cellular lysis was accomplished by the addition of SDS to a final concentration of 1% in the presence of 1M NaCl, after which almost all of the high molecular weight chromosomal DNA was preferentially precipitated by high gravity centrifugation, leaving the plasmid DNA molecules in the supernatant ('cleared lysate') (Guerry *et al*., 1973).

The enriched plasmid DNA molecules in the cleared lysate were then concentrated by quantitatively precipitating them with 10% PEG 6000 in the presence of 1M NaCl (Humphreys *et al*., 1975). The suspension was incubated for 2 - 3 hours at 0°C to ensure complete precipitation of the plasmid DNA molecules. After collecting the precipitate by centrifugation, it was

gently but thoroughly resuspended in a small volume of TE buffer. This was accomplished by swirling the pellet gently in the buffer and pipetting the suspension up and down several times with a 10 ml pipette until a clear solution was obtained. During the pipetting action, care was taken to avoid the formation of foam in the solution.

The ccc plasmid DNA molecules were finally separated from any remaining open circular and linear molecules by equilibrium centrifugation in CsCl containing EtBr (Radloff _et al_.,1967). During this process, EtBr binds by intercalating between the DNA base pairs and in so doing causes the DNA duplex to unwind. The ccc plasmid DNA molecules, having no free ends, can only unwind to a limited extent thus limiting the amount of EtBr bound. In contrast, the open circular and linear DNA molecules have no such topological constraints and can therefore bind more of the EtBr molecules. Because the density of the DNA/EtBr complex decreases as more EtBr is bound, and because more EtBr can be bound to the open circular and linear molecules than the ccc DNA molecules, the ccc plasmid DNA molecules have a higher density at saturating concentrations of EtBr. At the end of the isopynic centrifugation, a lower band in the CsCl-EtBr gradient representing the denser ccc _Thermus_ plasmid DNA molecules was visualized, and was collected with a hypodermic needle. A second CsCl-EtBr equilibrium

centrifugation was then performed to obtain pure Thermus plasmid DNA. The plasmid band was collected as before, extracted with isopropanol to remove the EtBr, and dialysed extensively against TE buffer. The DNA concentration was then determined.

Using this procedure, about 0.2 - 0.4 mg of ccc plasmid DNA was extracted from 1,000 ml of *T. aquaticus* B1 and B2, (about 8.5 - 9.0 gm wet weight).

4.3 Discussion

A method was developed to isolate pure ccc plasmid DNA molecules from *T. aquaticus* strains. This method was actually an extension of a number of common techniques used in the isolation of plasmids from a variety of bacterial cells, including *E. coli* and *B. subtilis*. The key step was the improved lysis procedure which used a preliminary dilute detergent wash prior to lysozyme treatment, and brought about rapid and complete lysis of the Thermus cells to ensure reproducible recovery of intact plasmids. After lysis, the ccc plasmid DNA was enriched and concentrated before being separated as a distinct band in the CsCl-EtBr density equilibrium centrifugation.

During the course of this study, Hishinuma *et al.* (1978) reported the presence of ccc plasmids in *T. flavus* and *T. thermophilus*. The method used by them to isolate the plasmids from these Thermus species depended on isotopic labelling of the bacterial DNA followed by CsCl-EtBr isopynic density

gradient centrifugation, and collection of fractions from the gradient. This method is invariably more tedious than the procedure developed here as it involves fraction collection and measurement of the radioactivity in each fraction. I was unable to compare the yield of plasmid DNA by their method as it was not mentioned. Using the procedure described in this Chapter, about 0.2 - 0.4 mg of ccc plasmid DNA was extracted from 1,000 ml of *T. aquaticus* B1 and B2, comparable to the yield of those plasmids whose copy numbers per cell were between 5 - 9, e.g. pAB118A, pAB118B and pAB124 (Bingham, 1980).

An observation made during the preparation of *Thermus* plasmids, particularly from *T. aquaticus* B1, was that sometimes the yield was very poor, almost negligible. This variability was thought to reflect cultural variability rather than the failure of the method to isolate plasmids. Very likely, the single colony of *T. aquaticus* B1 which was used to inoculate the liquid culture for plasmid preparation had lost the plasmid DNA. This is not very surprising as plasmids are generally considered to be dispensable to the host cells. Cultural variability is quite commonly encountered among thermophiles and is in fact one of the major problems with thermophiles, contributing to the snail's pace of progress in this field (Johnson, 1979).

To overcome this problem, before CsCl was added to the

DNA solution, prepared by dissolving the PEG-precipitated DNA in a small volume of TE buffer, samples were removed and examined by an agarose gel electrophoresis for the presence of a fast-moving ccc plasmid band (Fig. 9). Once the presence of the plasmid band was confirmed, the DNA solution was then centrifuged in CsCl-EtBr gradient.

In summary, a method was developed and is now available for the preparation of pure ccc plasmid DNA molecules from _T. aquaticus_.

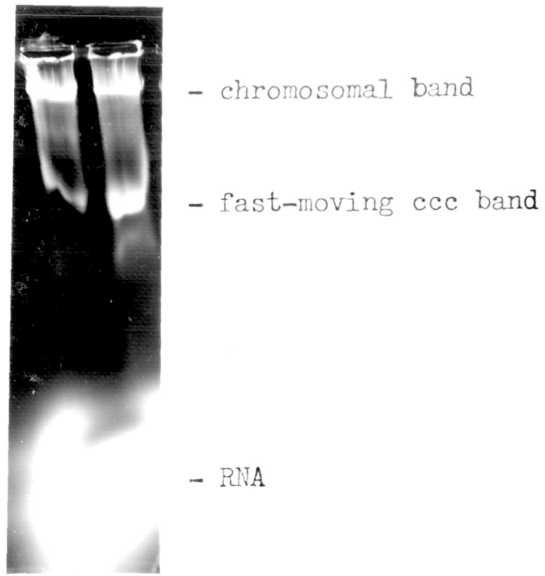


Fig. 9. Agarose gel electrophoresis (0.5%) of DNA solution of *T. aquaticus* B1, just before CsCl-EtBr isopynic density gradient centrifugation.

CHAPTER FIVE

CHARACTERIZATION OF PLASMIDS FROM *THERMUS* SPECIES

5.1 Introduction

It has now been established by physical means that there are ccc plasmids in some *T. aquaticus* strains. It would be very useful indeed, genetically, if certain phenotypic traits could be associated with their presence in these strains. A plasmid that carries an identifiable phenotypic trait would greatly facilitate studies on its transfer, and also be essential for developing a cloning system in *Thermus*. The *Thermus* strains which harbour them were therefore examined for phenotypic traits usually associated with plasmids in other microorganisms, such as resistance to antibiotics and production of bacteriocin. If they were cryptic plasmids, like those found in *T. flavus* and *T. thermophilus* (Hishinuma *et al*., 1978), it would be desirable to characterize them by restriction endonuclease mapping. A knowledge of their restriction endonuclease cleavage sites would allow us to construct *in vitro* hybrid replicons that carry selectable markers, or to map their replication origins.

5.2 Test for antibiotic sensitivity

The three plasmid-harbouring strains of *T. aquaticus* were

tested for resistance to several different antibiotics using Oxoid 'Multodisks' as described in Chapter Two (Section 2.10), and the results are shown in Table 9. In no case was any specific resistance detected, except in _T. aquaticus_ H which showed resistance to sulphafurazole. However, this was later shown to be a false resistance. Sulphafurazole acts like a sulphonamide which serves as a competitive substrate against p-aminobenzoate. But when the growth medium contains an adequate supply of p-aminobenzoate, as in the case of the Ramaley and Hixson medium (Section 2.4) which contains 0.2% tryptone and 0.2% yeast extract, then the effect of sulphafurazole is nullified. The growth inhibitory effect of sulphafurazole became apparent when _T. aquaticus_ H was inoculated on plates containing several concentrations of sulphafurazole and a lower concentration of tryptone (0.1%) and yeast extract (0.1%) in the medium (Table 10). This inhibitory effect became markedly obvious when _T. aquaticus_ H was streaked onto chemically defined minimal plates (Medium 162 + glucose + ammonium chloride, Section 2.4) with lower concentrations of sulphafurazole (Table 10). Thus, _T. aquaticus_ H was also found to be sensitive to sulphafurazole although it was more resistant than the strains B1 and B2 in the normal growth medium, i.e. Ramaley and Hixson medium.

The pattern of antibiotic inhibition of these strains was similar to that observed by Pask-Hughes and Williams (1977) who reported that the Icelandic _Thermus_ strains were sensitive to

TABLE 9

SENSITIVITY TO ANTIBIOTICS

Antibiotic	Dose (μg)	*T. aquaticus* strain B1	B2	H
Ampicillin	25	3+	3+	3+
Cephaloridine	25	3+	3+	3+
Chloramphenicol	50	3+	3+	3+
Colistin sulphate	10	3+	3+	2+
Erythromycin	50	3+	3+	3+
Kanamycin	30	3+	3+	3+
Nalidixic acid	30	3+	3+	3+
Nitrofurantoin	200	3+	3+	3+
Penicillin G	5[@]	3+	3+	3+
Streptomycin	25	3+	3+	3+
Sulphafurazole	500	3+	3+	-
Tetracycline	50	3+	3+	3+

[@] 5 units

- : No zone of inhibition

2+ : Zone of inhibition greater than 6mm but less than 12mm from the edge of the disc

3+ : Zone of inhibition greater than 12mm from the edge of the disc

TABLE 10

SENSITIVITY OF *T. AQUATICUS* H TO SULPHAFURAZOLE (SF)

MEDIUM	GROWTH
C.S. + 0.1% T. + 0.1% Y.E.	+++
" + " + " + SF (100)[@]	+
" + " + " + SF (250)	-
" + " + " + SF (500)	-
M162 + Glucose + A.C.	+++
" + " + " + SF (50)	-
" + " + " + SF (100)	-
" + " + " + SF (200)	-

C.S. : Castenholtz salts
T. : Tryptone
Y.E. : Yeast extract
M162 : Medium 162
A.C. : Ammonium chloride
@ : SF concentration in µg/ml
+++ : Good growth
\+ : Poor growth
\- : No growth

a variety of antibiotics, just like all the other _Thermus_ species known (Brock and Freeze, 1969; Williams, 1975; Hishinuma _et al._, 1978).

5.3 Assay for bacteriocin production

The ability of the three plasmid-bearing _T. aquaticus_ strains to produce bacteriocin was examined by the double-layer technique of Fredericq (1957) (Chapter Two, Section 2.11), using the other nonplasmid-harbouring _Thermus_ strains as indicators. No clear zone of inhibition was observed around the three colonies, indicating that the presence of plasmids could not be correlated with bacteriocin production.*

This result was similar to that reported by Hishinuma _et al._ (1978) on the plasmid-harbouring strains of _T. flavus_ and _T. thermophilus_.

5.4 Restriction endonuclease mapping

The plasmids isolated from _T. aquaticus_ B1 and B2 were analysed by restriction endonuclease mapping, but those from _T. aquaticus_ H were not, as the strain was only available recently and the plasmids discovered towards the end of the project.

The strategy employed to map the plasmids by restriction

* To be absolutely sure that the presence of the plasmids is not correlated with bacteriocin production, it is necessary to perform the double-layer technique of Fredericq (1957) using _T. aquaticus_ B1, B2 and H strains cured of plasmids as the indicators.

endonuclease digestion was as follows: first, the plasmids were digested with a range of restriction endonucleases. Samples were then applied to an agarose gel and electrophoresed to determine the number of cleavage sites for each of the endonucleases, as well as to have an estimate of the sizes of the fragments generated, e.g. large, medium or small. Next, the sizes of the fragments were accurately determined by running them in a suitable concentration of agarose gel alongside of the appropriate DNA standards (Table 8, Section 2.25). The electrophoretic mobilities of the DNA fragments in the gel were measured as accurately as possible, and the sizes of the endonuclease--generated fragments determined graphically as described in Chapter Two (Section 2.25). The size of each plasmid could then be determined by adding up the sizes of all the fragments produced in each endonuclease digest.

Restriction endonucleases which cleaved the plasmid once were noted. Their cleavage sites in the plasmid were then positioned in relation to each other by using a double-digest technique, in which the plasmid was digested in turn by a pair of the enzymes. All the possible combinations of pairs of restriction endonucleases were used. The fragments produced were sized, and they could then be used to construct a physical map of the plasmid showing the relative positions of the cleavage sites of these endonucleases. To construct such a map, the same

principle used in the construction of a genetic map was employed, i.e. all map distances are additive. In other words, if the distance between two sites A and B were equal to the sum of the distances between A and C and C and B, then the order of these sites would be A - C - B.

Once these single cleavage sites had been positioned in the plasmids, they were in turn used as reference points to locate the sites of other restriction endonucleases which were found to cleave the plasmid into two or more fragments. This was achieved by a series of double-digests in which each of the single-cleavage-site restriction endonuclease was used in turn as one of the enzymes in a double-digest with a multiple-site restriction endonuclease. In this way, a composite physical map of the plasmid showing the relative positions of the various restriction endonuclease sites was constructed.

5.4.1 Restriction endonuclease cleavage map of pCLK1

pCLK1 is the plasmid isolated from *T. aquaticus* B1. The preliminary restriction endonuclease digestions (Table 11) showed that pCLK1 is not cleaved by *Bcl*I, *Eco*RI, *Hpa*I, *Hin*dIII, and *Xba*I; cleaved once by *Bgl*II, *Pst*I, *Sal*I, and *Xor*II ; twice by *Bst*EII and *Kpn*I ; three times by *Sst*II ; four times by *Hin*fI ; and seven times by *Bst*I. *Alu*I, *Ava*II, *Bgl*I, *Eco*RII, *Hae*II, *Hae*III, *Hpa*II, *Mbo*I, *Mbo*II, *Sma*I, *Sst*I, and *Taq*I were

all found to cleave pCLK1 into many fragments (6 or more, mostly small fragments) indicating that there are many of these sites within the plasmid pCLK1. From the sizes of the fragments determined, the average size of pCLK1 was ascertained to be 8.76 kb (Table 11).

Double-digests were then performed using the following combinations of restriction endonucleases, each of which was found to cleave the plasmid once : *Bgl*II - *Pst*I, *Bgl*II - *Sal*I, *Bgl*II - *Xor*II, *Pst*I - *Sal*I, and *Pst*I - *Xor*II, to determine the relative positions of these sites in the plasmid. The sizes of the fragments generated in these double-digests were determined and given in Table 12. From the fragment sizes produced in the following double-digests : *Bgl*II - *Pst*I, *Bgl*II - *Sal*I, and *Pst*I - *Sal*I, it could be ascertained that the order of these three sites is *Pst*I - *Bgl*II - *Sal*I, the distance between *Pst*I and *Sal*I (2.64 kb in one direction) being the sum of the distances between *Pst*I and *Bgl*II (2.38 kb) and *Bgl*II and *Sal*I (0.26 kb) in the same direction. Similarly, from the fragment sizes generated in the following combinations of double-digests: *Bgl*II - *Xor*II, *Bgl*II - *Pst*I, and *Pst*I - *Xor*II, it could be determined that the order of these three sites is *Xor*II - *Pst*I - *Bgl*II . The distance between *Xor*II and *Bgl*II (3.05 kb in one direction) was found to be the sum of the distances between *Xor*II and *Pst*I (0.70 kb) and *Pst*I and *Bgl*II (2.38 kb) in the

TABLE 11

Restriction Endonuclease Cleavage Sites in pCLK1

Enzyme	No.	Fragment sizes (kb) A	B	C	D	E	F	G	Sum of fragment sizes (kb)
AluI	m	ND							
AvaII	m	ND							
BclI	0								
BglI	m	ND							
BglII	1	8.75							8.75
BstI	7	2.38,	1.98,	1.85,	1.10,	0.85,	0.38,	0.22	8.76
BstEII	2	8.55,	0.20						8.75
EcoRI	0								
EcoRII	m	ND							
HaeII	m	ND							
HaeIII	m	ND							
HindIII	0								
HinfI	4	3.33,	3.12,	1.48,	0.82				8.75
HpaI	0								
HpaII	m	ND							
KpnI	2	6.67,	2.10						8.77
MboI	m	ND							
MboII	m	ND							
PstI	1	8.76							8.76
SalI	1	8.76							8.76
SmaI	m	ND							
SstI	m	ND							
SstII	3	6.04,	2.38,	0.34					8.76
TaqI	m	ND							
XbaI	0								
XorII	1	8.76							8.76

m : multiple sites (6 or more), generating many fragments

0 : no cleavage site

ND : not determined

TABLE 12

Fragments Produced By Digestion Of pCLK1 With Two Restriction Endonucleases Having One Cleavage Site Each

Enzyme Pair	Fragment sizes (kb)	Sum of fragment sizes (kb)
BglII - PstI	6.35 , 2.38	8.73
PstI - SalI	6.1 , 2.64	8.74
BglII - SalI	8.5 , 0.26	8.76
BglII - XorII	5.7 , 3.05	8.75
PstI - XorII	8.1 , 0.70	8.80

same direction. This second site-order of XorII -PstI - BglII confirms the first site-order of PstI - BglII - SalI, because in both cases the PstI site is to the left of the BglII site. Combining these two site-orders, the order of the four endonuclease sites is therefore as follows: XorII - PstI - BglII - SalI. A preliminary physical map of pCLK1 showing the relative positions of BglII, PstI, SalI and XorII cleavage sites is given in Fig. 10.

Once these single-cleavage sites had been positioned in pCLK1, they could be used as reference points to locate the sites of other restriction endonucleases which were found to cleave the plasmid more than once, such as BstEII, KpnI, SstII, HinfI, and BstI. A series of double endonuclease digestions were made involving the following combinations : KpnI - BglII, KpnI - Pst I, KpnI - XorII, BstEII - BglII, BstEII - PstI, BstEII - KpnI, SstII - PstI, SstII - SalI, HinfI - BglII, HinfI - PstI, HinfI - SalI, HinfI - KpnI, BstI - BglII, BstI - PstI, BstI - SalI, BstI - XorII, BstI - KpnI, BstI - SstII, and BstI - HinfI. Fragments produced in these double-digests were then systematically sized by agarose gel electrophoresis alongside of the appropriate DNA standards as described in Chapter Two (Section 2.25). Some of the gels used in the determination of the sizes of these fragments are shown in Fig. 11(a) - ( ). The fragment sizes produced in these double-

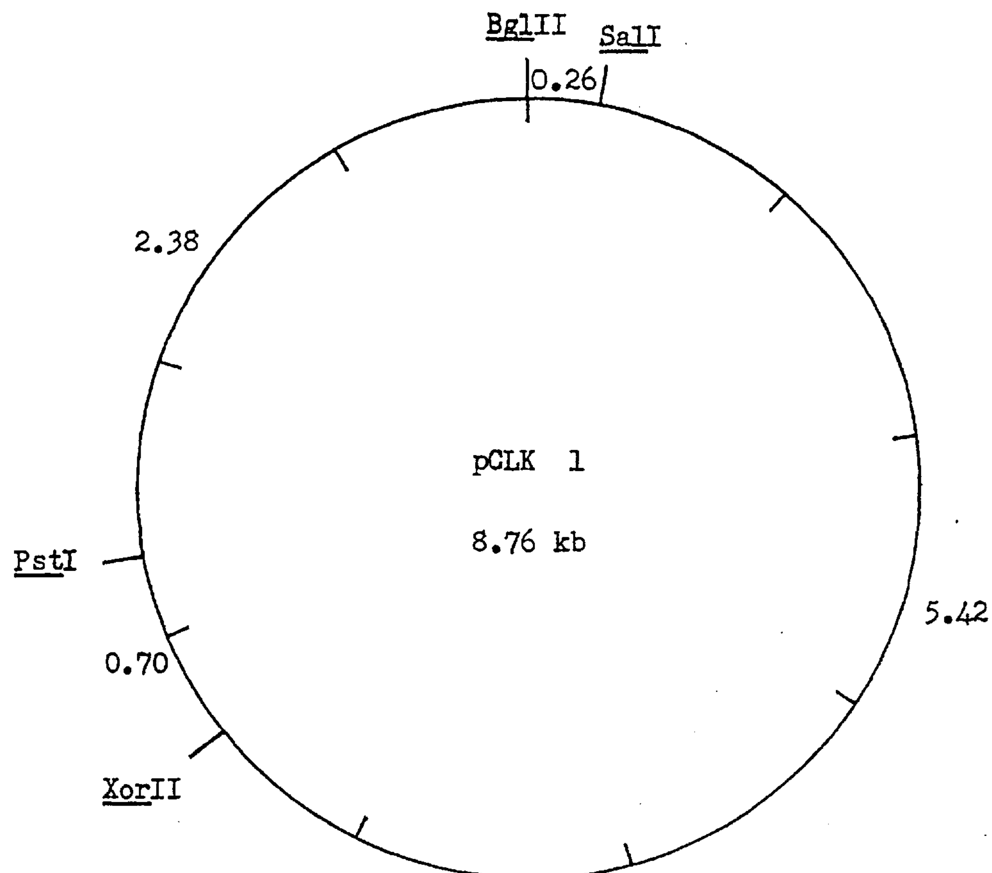


Fig. 10. The pCLK1 restriction endonuclease cleavage map, showing the positions of *BglII*, *PstI*, *SalI*, and *XorII*.

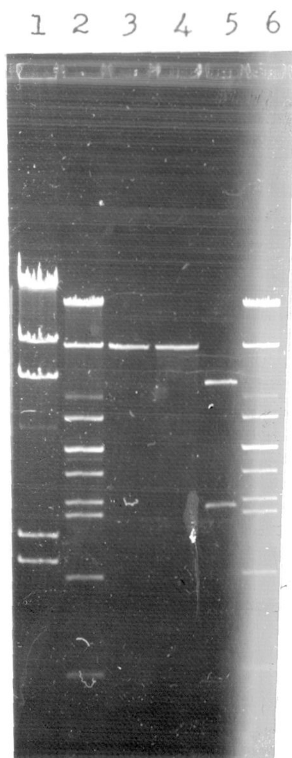


Fig. 11 (a). Agarose gel electrophoresis (0.7%) of pCLK1 digested with restriction endonucleases. (1) Reference lambda DNA digested with HindIII ; (2) & (6) reference lambda DNA digested with BstI and EcoRI ; (3) pCLK1 digested with SalI ; (4) pCLK1 cleaved with PstI ; (5) pCLK1 digested with PstI and SalI.

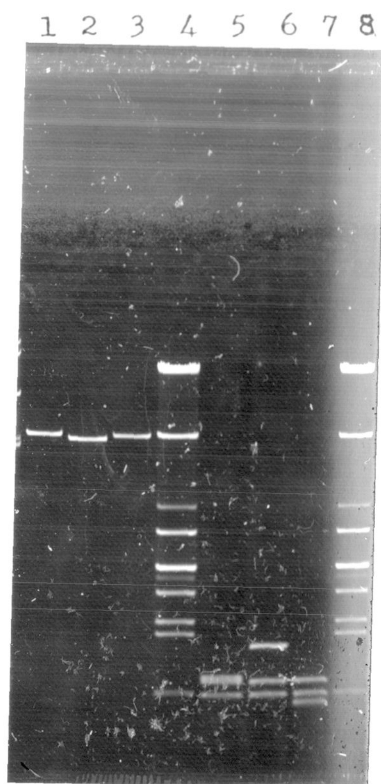


Fig. 11 (b). Agarose gel electrophoresis (0.5%) of pCLK1 digested with restriction endonucleases. (1) pCLK1 digested with BglII ; (2) pCLK1 digested with BglII and SalI ; (3) pCLK1 digested with SalI ; (4) and (8) reference lambda DNA digested with BstI and EcoRI ; (5) pCLK1 digested with BglII and BstI ; (6) pCLK1 digested with BstI ; (7) pCLK1 cleaved with BstI and SalI.

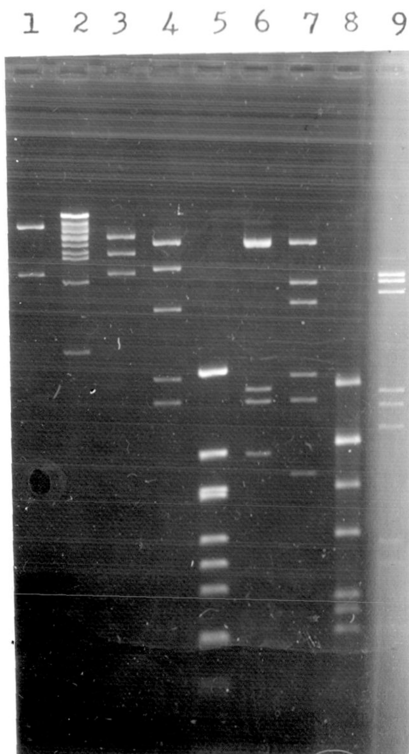


Fig. 11 (c). Agarose gel electrophoresis (2.0%) of pCLK1 digested with restriction endonucleases. (1) pCLK1 cleaved with KpnI and SalI ; (2) reference lambda DNA digested with BstI and EcoRI. pCLK1 digested with (3) KpnI and PstI , (4) PstI and HinfI ; (5) pBR322 digested with HinfI and EcoRI. pCLK1 digested with (6) HinfI and SalI , (7) HinfI and KpnI ; (8) pBR322 digested with AluI ; and (9) pCLK1 cleaved with BstI and KpnI.

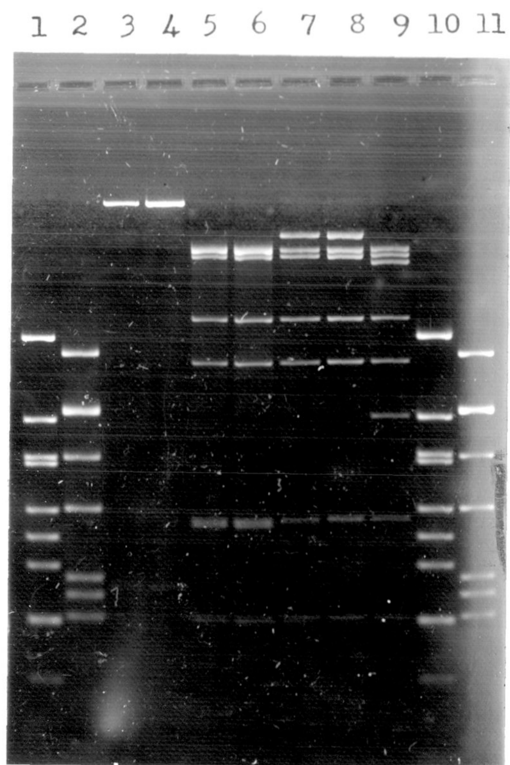


Fig. 11 (d). Agarose gel electrophoresis (2.0%) of pCLK1 digested with restriction endonucleases. (1) & (10) pBR322 cleaved with HinfI and EcoRI ; (2) & (11) pBR322 cleaved with AluI. pCLK1 cleaved with (3) & (4) BglII and SalI ; (5) & (6) BglII and BstI ; (7) & (8) BstI ; and (9) BstI and SalI.

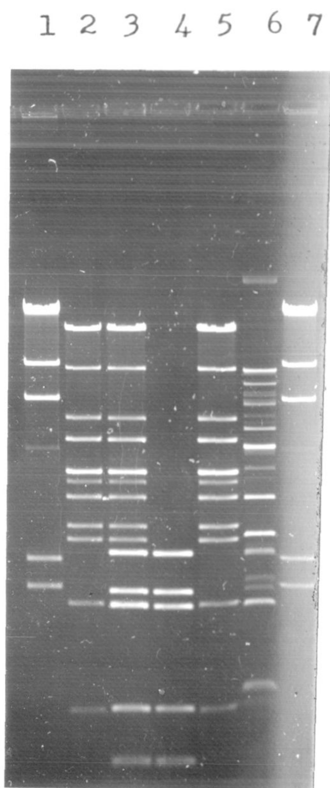


Fig. 11 (e). Agarose gel electrophoresis (0.7%) of pCLK1 digested with restriction endonucleases. (1) & (7) Reference lambda DNA cleaved with HindIII ; (2) & (5) reference lambda DNA digested with BstI and EcoRI ; (3) lambda DNA cleaved with BstI and EcoRI plus pCLK1 digested with BstI ; (4) pCLK1 digested with BstI ; and (6) pCLK1 cleaved with BstI (partial digestion).

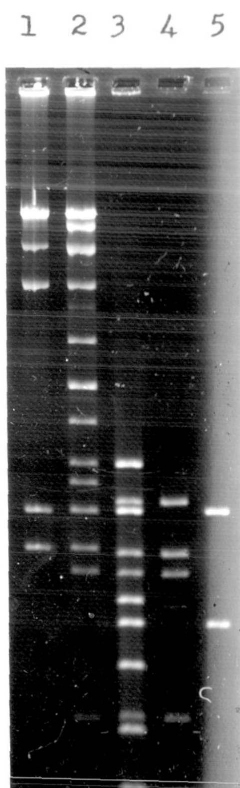


Fig. 11 (f). Agarose gel electrophoresis (1.0%) of pCLK1 digested with restriction endonucleases. (1) Lambda DNA digested with HindIII ; (2) lambda DNA digested with BstI and EcoRI plus lambda DNA cleaved with HindIII ; (3) pCLK1 digested with BstI plus ØX174 cleaved with HpaII plus ØX174 cleaved with HaeII plus ØX174 cleaved with HaeIII ; (4) pCLK1 cleaved with BstI ; (5) ØX174 digested with HaeII.

digests are shown in Table 13.

The data in the first group indicate that KpnI cleaves pCLK1 into two fragments : fragment A (6.67 kb) and fragment B (2.1 kb), and the three single-cleavage sites of BglII, PstI, and XorII are located within the large fragment A. It can be seen from the data that of the three known cleavage sites, i.e. XorII - Pst I - BglII, the BglII site is the nearest to one of the KpnI sites, cleaving the fragment A of KpnI digest into two fragments of 6.36 kb and 0.31 kb. The site-order of these restriction endonucleases can therefore be derived as XorII - PstI - BglII - KpnI. The other KpnI site is 2.1 kb away from the first KpnI site, to its right. An improved restriction endonuclease cleavage map of pCLK1 showing the positions of the KpnI sites relative to the BglII, PstI, SalI and XorII sites can therefore be drawn (Fig. 12a).

Similarly, the data in the second group indicate that all the BglII, PstI, and KpnI sites lie within the large fragment A released by BstEII digestion. At the same time, it can be seen that the two BstEII sites are within the small fragment B of KpnI digest. Accordingly, the cleavage map of pCLK1 in Fig. 12a was improved to include the relative positions of the two BstEII sites (Fig. 12b).

From the third group of data, it can be seen that the

TABLE 13

Fragments Produced By Double Digestions Of pCLK1

Enzyme pair	Fragment sizes (kb) A B C D E F G H I J	Sum of fragment sizes (kb)
1) KpnI	6.67,2.1	8.77
"-BglII	6.36,2.1 ,0.31	8.77
"-PstI	3.97,2.7 ,2.1	8.77
"-XorII	3.40,3.25,2.1	8.75
2) BstEII	8.55,0.20	8.75
"-BglII	8.15,0.44,0.20	8.79
"-PstI	5.73,2.80,0.20	8.73
"-KpnI	6.67,1.80,0.20,(0.1)	8.77
3) SstII	6.04,2.38,0.34	8.76
"-PstI	3.70,2.38,2.38,0.34	8.8
"-SalI	6.04,2.20,0.34,0.22	8.8
4) HinfI	3.33,3.12,1.48,0.82	8.75
"-BglII	3.33,3.12,0.88,0.82,0.60	8.75
"-PstI	3.33,2.20,1.48,0.92,0.82	8.75
"-SalI	3.33,3.12,0.86,0.82,0.62	8.75
"-KpnI	3.12,1.80,1.53,0.94,0.82,0.54	8.75
5) BstI	2.38,1.98,1.85,1.10,0.85,0.38,0.22	8.76
"-BglII	2.01,1.98,1.85,1.10,0.85,0.38,0.37,0.22	8.76
"-PstI	2.38,1.98,1.80,1.10,0.85,0.38,0.22,(0.05)	8.76
"-SalI	1.98,1.85,1.76,1.10,0.85,0.62,0.38,0.22	8.76
"-XorII	2.38,1.85,1.32,1.10,0.85,0.68,0.38,0.22	8.76
"-KpnI	1.98,1.85,1.68,0.85,0.79,0.70,0.38,0.33,0.22	8.78
"-SstII	1.98,1.90,1.85,0.85,0.48,0.43,0.38,0.34,0.33,0.22	8.76
"-HinfI	1.98,1.26,1.10 (x2),1.00,0.86,0.82,0.22,0.20,0.18,(0.04)	8.76

(size) : DNA fragment not detected in the gel, but can be predicted from the sum of the fragment sizes

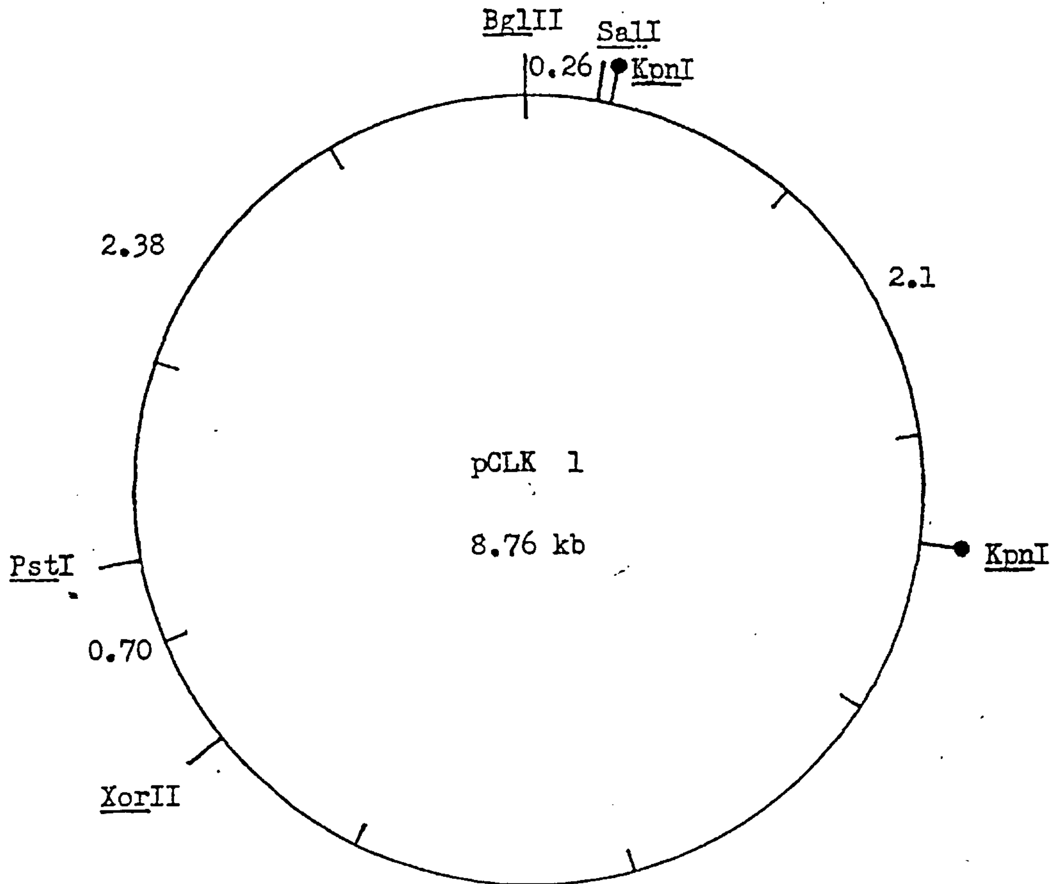


Fig. 12a. The pCLK1 restriction endonuclease cleavage map, showing the positions of BglII, KpnI, PstI, SalI, and XorII.

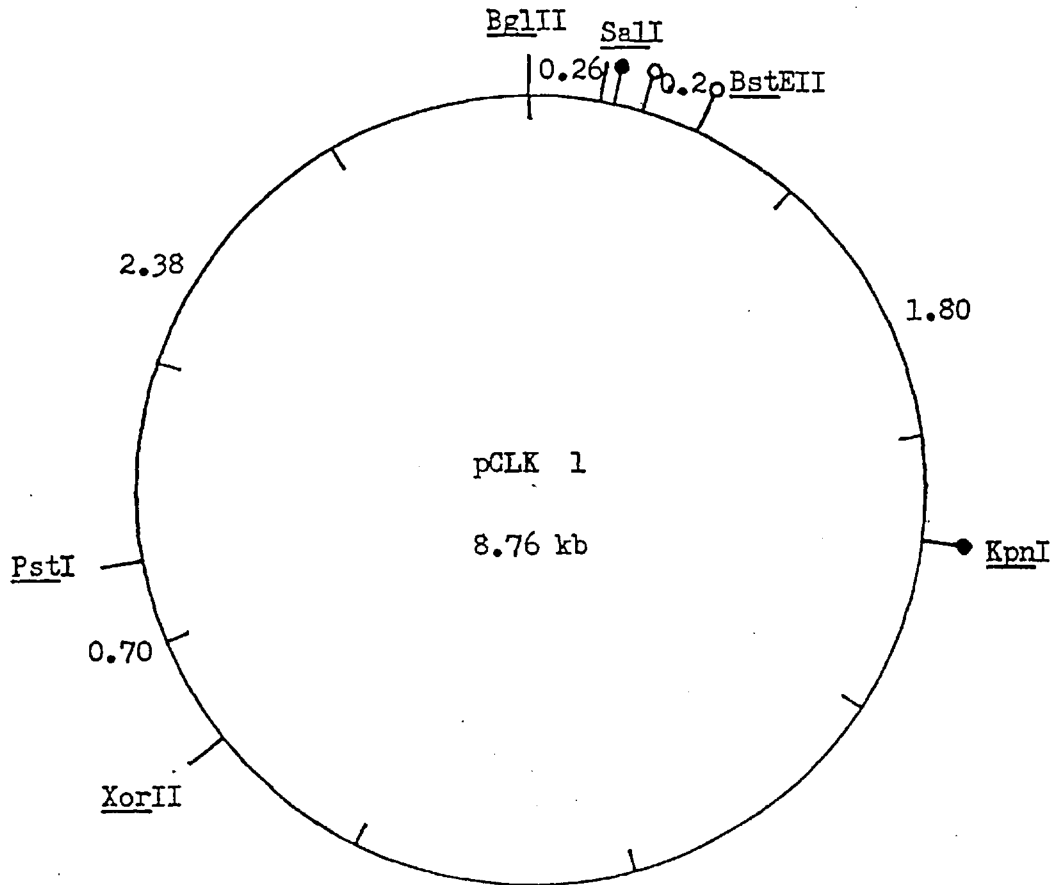


Fig. 12b. The pCLK1 restriction endonuclease cleavage map, showing the positions of BglII, BstEII, KpnI, PstI, SalI, and XorII.

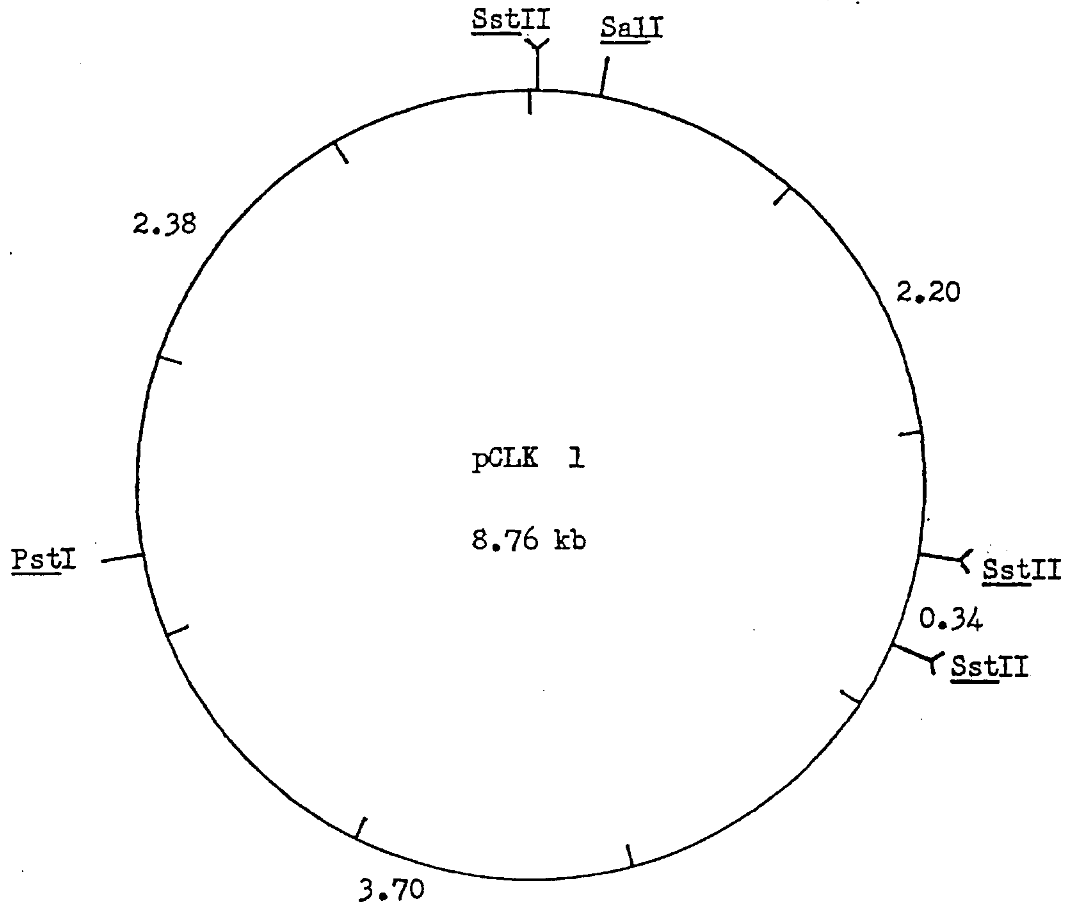


Fig. 12c. The pCLK1 restriction endonuclease cleavage map, showing the positions of *PstI*, *SalI*, and *SstII*.

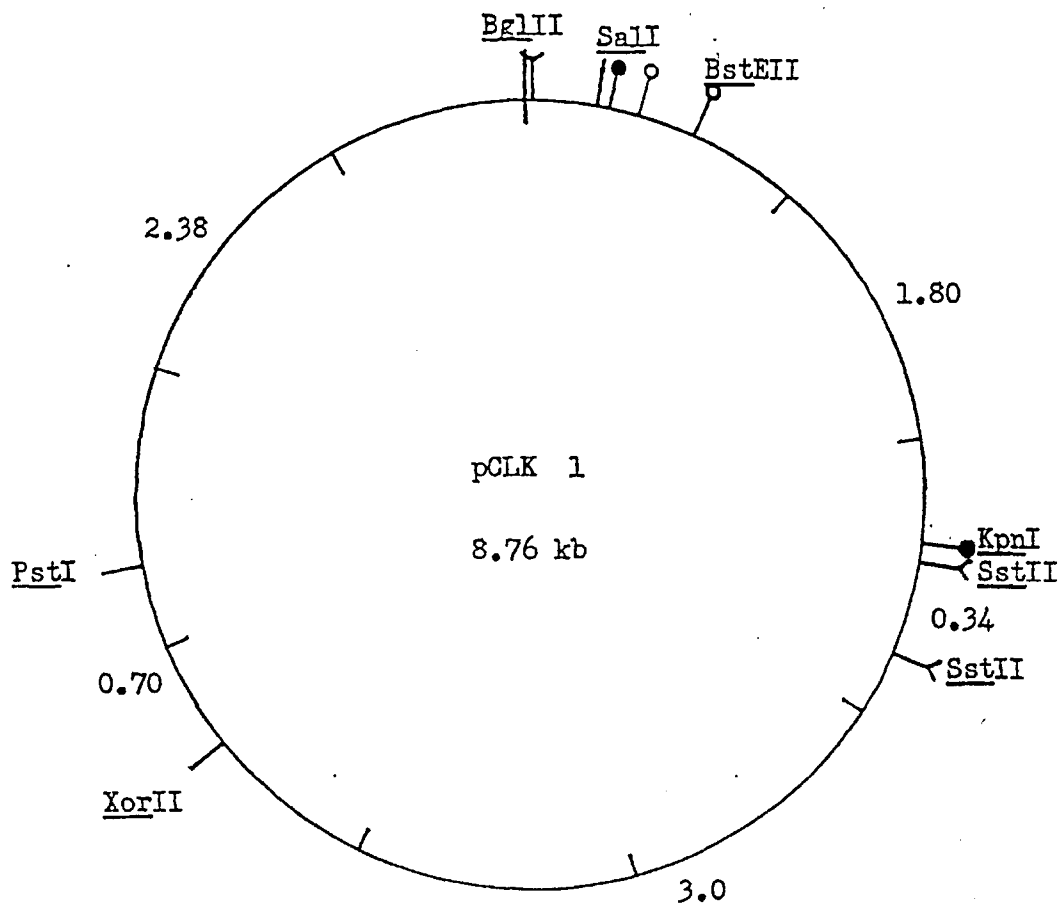


Fig. 12d. The pCLK1 restriction endonuclease cleavage map, showing the positions of BglII, BstEII, KpnI, PstI, SalI, SstII, and XorII.

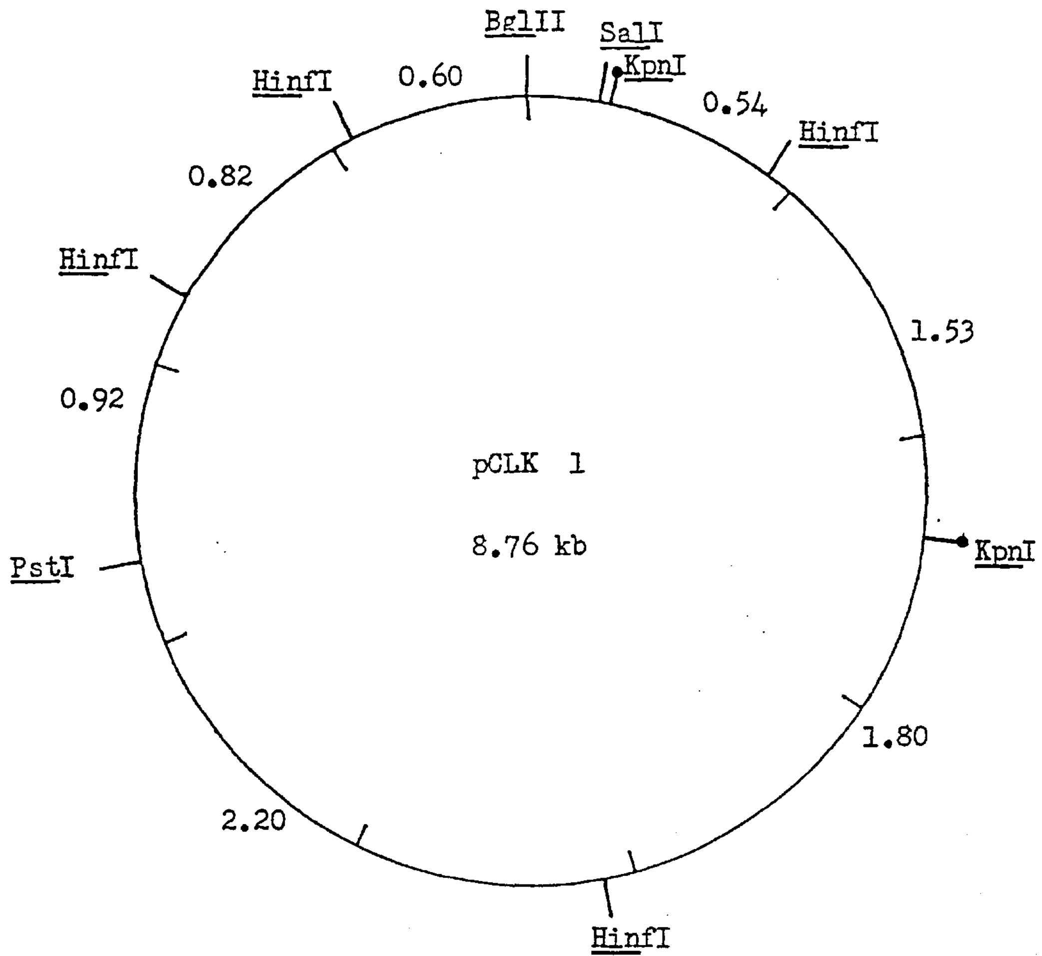


Fig. 12e. The pCLK1 restriction endonuclease cleavage map, showing the positions of BglII, HinfI, KpnI, PstI, and SalI.

PstI site is positioned within the fragment A of SstII digest, while the SalI site is located in the fragment B of SstII digest. The smallest fragment (C, 0.34 kb) produced by the SstII digestion was observed not to be cleaved by either of these enzymes. It must therefore lie between these two known sites. Applying the principle of addition, a cleavage map of pCLK1 depicting the relative positions of these five sites was constructed (Fig. 12c). This map was then superimposed onto the previous cleavage map (Fig. 12b) to produce the composite cleavage map of pCLK1 in Fig. 12d.

Similarly, an analysis of the group four data indicates that both the BglII and SalI cleavage sites lie within the fragment C generated by HinfI digestion. The PstI site is within the fragment B of HinfI digest, while the two KpnI sites lie within the fragment A and the fragment C of HinfI. As the relative positions of the BglII, KpnI, PstI, and SalI sites were already known, it was not difficult to deduce the positions of the four HinfI sites. A map relating the positions of these HinfI sites to the known BglII, KpnI, PstI, and SalI sites in the plasmid pCLK1 is shown in Fig. 12e.

The last group of the double-digest data shows that the single-cleavage sites of BglII and SalI lie within the fragment A of BstI; the PstI site is located within the fragment C of

BstI; the single XorII site is positioned in the fragment B of BstI; and the two KpnI sites are distributed within the fragments A and D of BstI. From the sizes of the fragments known so far and the previous knowledge of the positions of the BglII, KpnI, PstI, SalI, and XorII sites, it could be deduced that the fragment A of BstI is linked to the fragment D in one direction, and to the fragment C in the opposite direction, separated by the smallest fragment G of BstI (0.22 kb). The other end of the fragment C is joined to the fragment B of BstI. Thus, the orientation of the BstI fragments known so far is B - C - G - A - D (Fig. 12f).

The BstI - SstII double-digest indicates that SstII cleaves the fragments A and D of BstI. The smallest fragment C of SstII digest (0.34 kb) was produced intact, indicating that this fragment lies within either the fragment A or D of BstI digest. From the known positions of the SstII sites in pCLK1, it could be derived that the fragment C of SstII is in fact located in the fragment D of BstI. This result also confirms the previous finding that the fragments A and D of BstI are linked together at one end. The last double-digest of BstI - HinfI demonstrates that HinfI cleaves the following fragments of BstI digest : A, C, and F. Again here, the smallest fragment of HinfI digest (0.82 kb) was produced intact, indicating that it lies within either the fragment A or C of BstI digest. Having known the

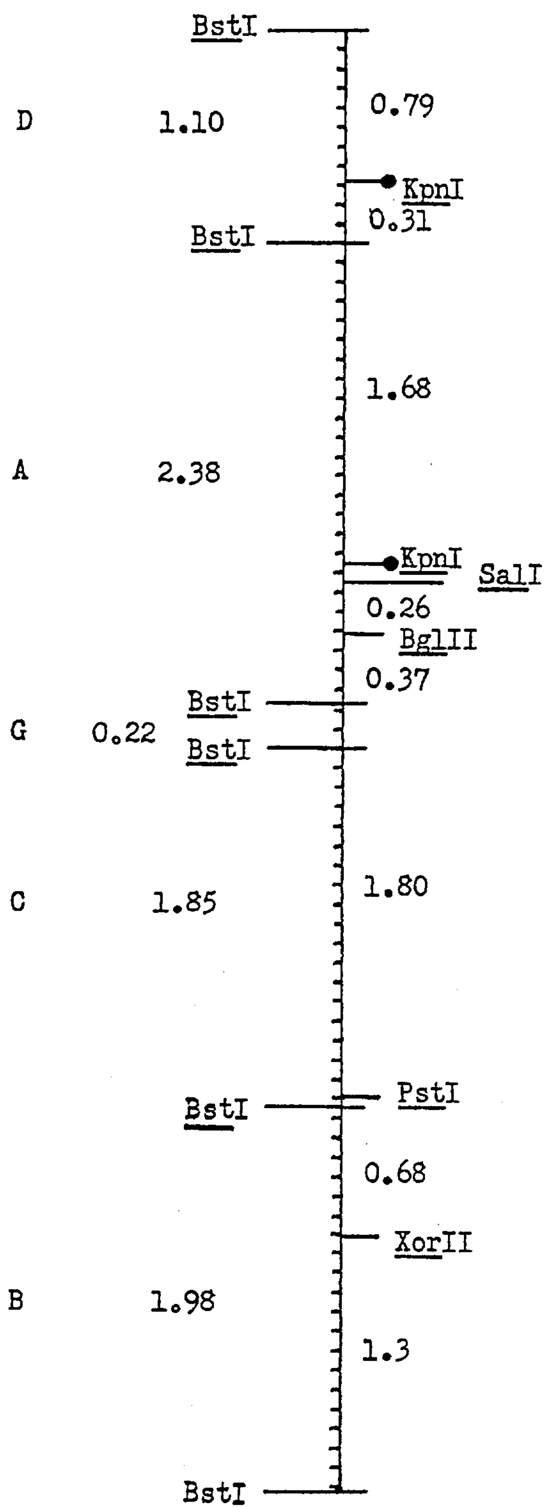


Fig. 12f. The partial cleavage map of pCLK1, showing the orientation of the BstI fragments D, A, G, C and B.

positions of the four HinfI sites in pCLK1 and the orientation of some of the BstI fragments (i.e. B - C - G - A - D), it was not difficult to decide that the fragment D of HinfI (0.82 kb) is positioned within the fragment C of BstI (1.85 kb). The most useful observation from this double-digest was that one of the HinfI sites lies within the fragment F of BstI. This observation allows the positions of the two remaining BstI fragments, E and F, in the plasmid to be resolved. The fragment F of BstI was found to be joined to the fragment B, while the fragment E was found to be between the fragments F and D of BstI.

The final composite restriction endonuclease cleavage map of pCLK1 showing the relative positions of the sites of the nine endonucleases is shown in Fig. 13. This, I believe, is the first known physical restriction endonuclease cleavage map of a plasmid from the extreme thermophile *T. aquaticus*.

5.4.2 Restriction endonuclease cleavage map of pCLK2 and pCLK3

The extrachromosomal ccc DNA isolated from *T. aquaticus* B2 was shown by agarose gel electrophoresis to comprise two molecular species of plasmid DNA : pCLK2 (the larger plasmid) and pCLK3 (the smaller plasmid) (Fig. 14). The presence of two plasmid species in the same DNA sample made the analyses by restriction endonuclease digestion more difficult, but not

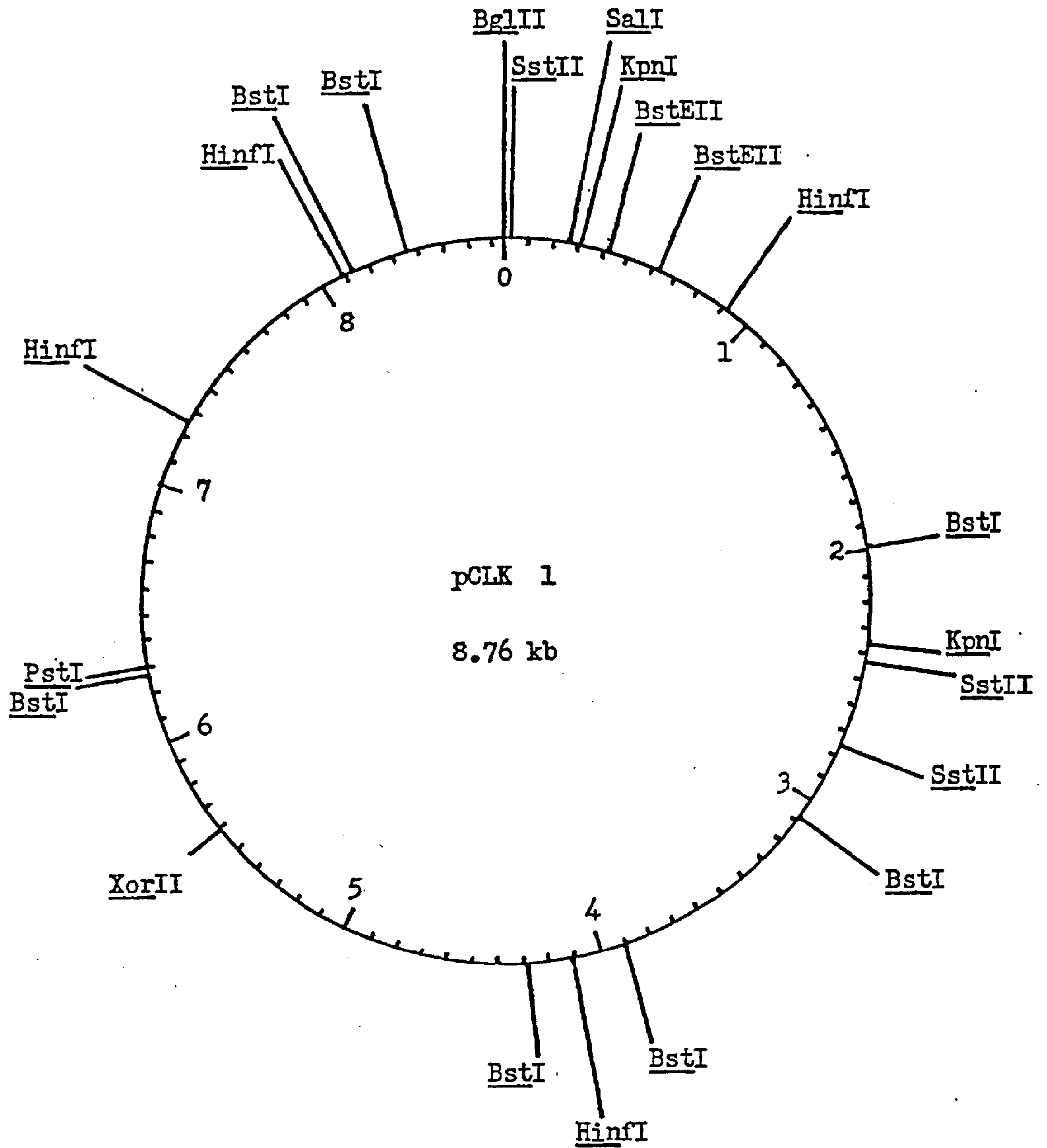


Fig. 13. The pCLK 1 restriction endonuclease cleavage map

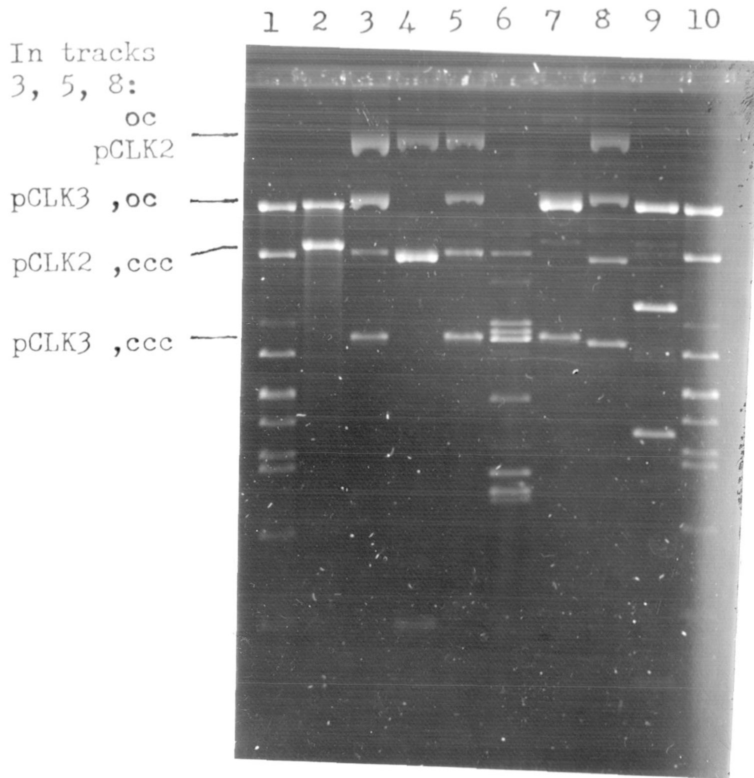


Fig. 14. Agarose gel electrophoresis (0.5%) of pCLK2 and pCLK3 treated with restriction endonucleases. (1) and (10) Lambda DNA cleaved with BstI and EcoRI. pCLK2 and pCLK3 treated with (2) PstI ; (3) BclI ; (4) BstEII ; (5) HpaI ; (6) SstII ; (7) XbaI ; (8) XorII ; and (9) HindIII.

impossible.

The preliminary restriction endonuclease digestions (Table 14) showed that both pCLK2 and pCLK3 are not cleaved by BclI, BglII, EcoRI, HpaI, SalI, and XorII. Both plasmids were observed to be cleaved by AluI, AvaII, BglI, BstI, EcoRII, HaeII, HaeIII, HinfI, HpaII, MboI, MboII, SmaI, SstI, SstII, and TaqI into many fragments. PstI was found to cleave each plasmid once. BstEII does not cleave pCLK2 but cleaves pCLK3 twice into two fragments. HindIII cleaves pCLK2 once and pCLK3 three times to produce three fragments. XbaI cleaves pCLK2 once but does not cleave pCLK3. From the sizes of the fragments determined, the average size of pCLK2 was determined to be 17.24 kb, and that of pCLK3, 9.95 kb (Table 14). One of the agarose gels used in the determination of the sizes of these fragments is shown in Fig. 14.

The double-digests of HindIII - PstI, HindIII - XbaI, and PstI - XbaI were performed to locate the relative positions of these three sites in pCLK2. The sizes of the fragments generated in these double-digests were determined and given in Table 15. From the fragment sizes produced, it could be ascertained that the order of the cleavage sites of these three enzymes is PstI - XbaI - HindIII, the distance between PstI and HindIII being the sum of the distances between PstI

TABLE 14

Restriction Endonuclease Cleavage Sites in pCLK2 And pCLK3

Enzyme	No. in pCLK2	No. in pCLK3	Fragment sizes (kb)	Sum of fragment sizes (kb) pCLK2	Sum of fragment sizes (kb) pCLK3
AluI	m	m	ND		
AvaII	m	m	ND		
BclI	0	0			
BglI	m	m	ND		
BglII	0	0			
BstI	m	m	ND		
BstEII	0	2	8.90, 1.05	-	9.95
EcoRI	0	0			
EcoRII	m	m	ND		
HaeII	m	m	ND		
HaeIII	m	m	ND		
HindIII	1	3	17.24, 6.20, 3.05, 0.71	17.24	9.95
HinfI	m	m	ND		
HpaI	0	0			
HpaII	m	m	ND		
MboI	m	m	ND		
MboII	m	m	ND		
PstI	1	1	17.24, 9.95	17.24	9.95
SalI	0	0			
SmaI	m	m	ND		
SstI	m	m	ND		
SstII	m	m	ND		
TaqI	m	m	ND		
XbaI	1	0	17.24	17.24	-
XorII	0	0			

m : multiple sites, generating many fragments

0 : no cleavage site

ND : not determined

TABLE 15

Fragments Produced By Double Digestions Of pCLK2

Enzyme pair	Fragment sizes (kb)	Sum of fragment sizes (kb)
HindIII - PstI	12.20, 5.05	17.25
HindIII - XbaI	15.20, 2.04	17.24
PstI - XbaI	14.20, 3.02	17.22

and *XbaI* and *XbaI* and *HindIII* in the same direction (i.e. 5.05 kb = 3.02 kb + 2.04 kb). A simple restriction endonuclease cleavage map of pCLK2 showing the relative positions of the *PstI*, *XbaI*, and *HindIII* sites was constructed as shown in Fig. 15.

Similarly, the following double-digests of *BstEII* - *HindIII*, *BstEII* - *PstI*, and *HindIII* - *PstI* were performed to locate the relative positions of these sites in pCLK3. The fragment sizes generated in these double-digests are given in Table 16. The first group of data shows that the *PstI* site lies within the large fragment A of *BstEII* digest, cleaving it into two new fragments of 5.95 kb and 2.95 kb. The second group of data indicates that the single-cleavage site of *PstI* is located in the fragment B of *HindIII* digest; the three *HindIII* sites are found within the fragment A of *BstEII* digest; and the two *BstEII* sites are positioned in the fragment A of *HindIII* digest. Again, using the basic principle that the map distances between sites are additive, a physical restriction endonuclease cleavage map showing the relative positions of these sites in pCLK3 was constructed (Fig. 16).

Some of the agarose gels used in the elucidation of the restriction endonuclease cleavage maps of pCLK2 and pCLK3 are shown in Fig. 17(a) - (c).

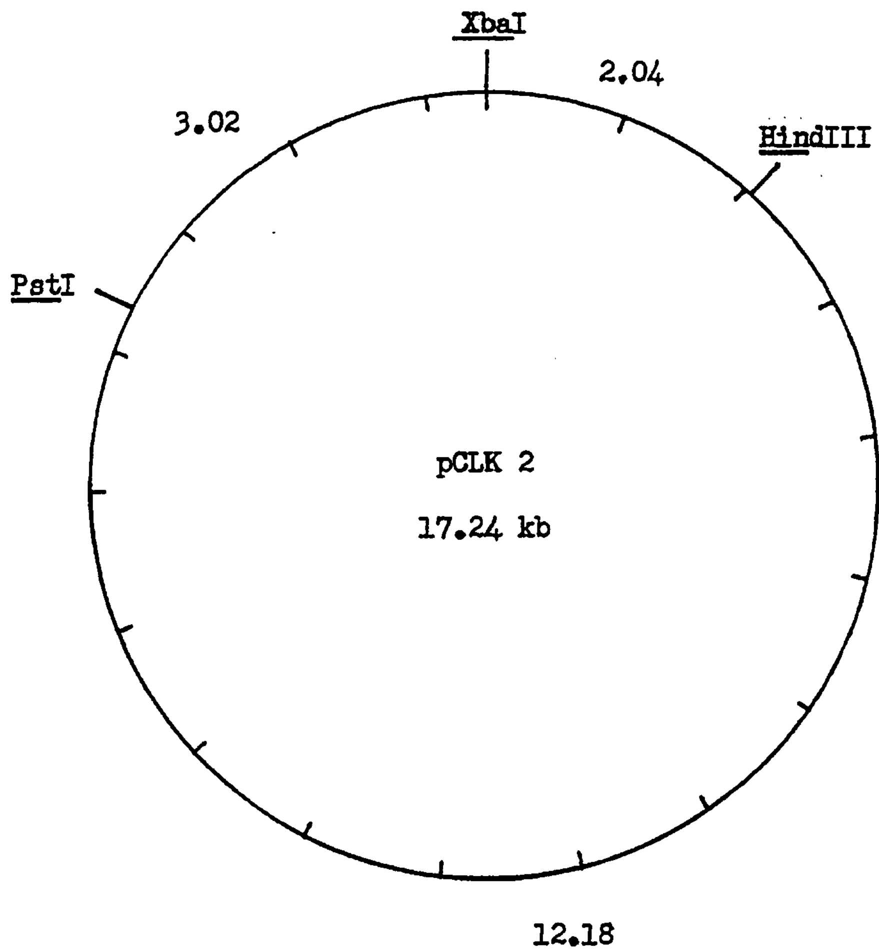


Fig. 15. The pCLK2 restriction endonuclease cleavage map.

TABLE 16

Fragments Produced By Double Digestions Of pCLK3

	Enzyme pair	Fragment sizes (kb)					Sum of fragment sizes (kb)
		A	B	C	D	E	
1)	BstEII	8.90,	1.05				9.95
	BstEII - PstI	5.95,	2.95,	1.05			9.95
2)	HindIII	6.20,	3.05,	0.71			9.96
	HindIII - PstI	6.20,	1.59,	1.45,	0.71		9.95
	HindIII - BstEII	4.50,	3.05,	1.05,	0.71,	0.65	9.95

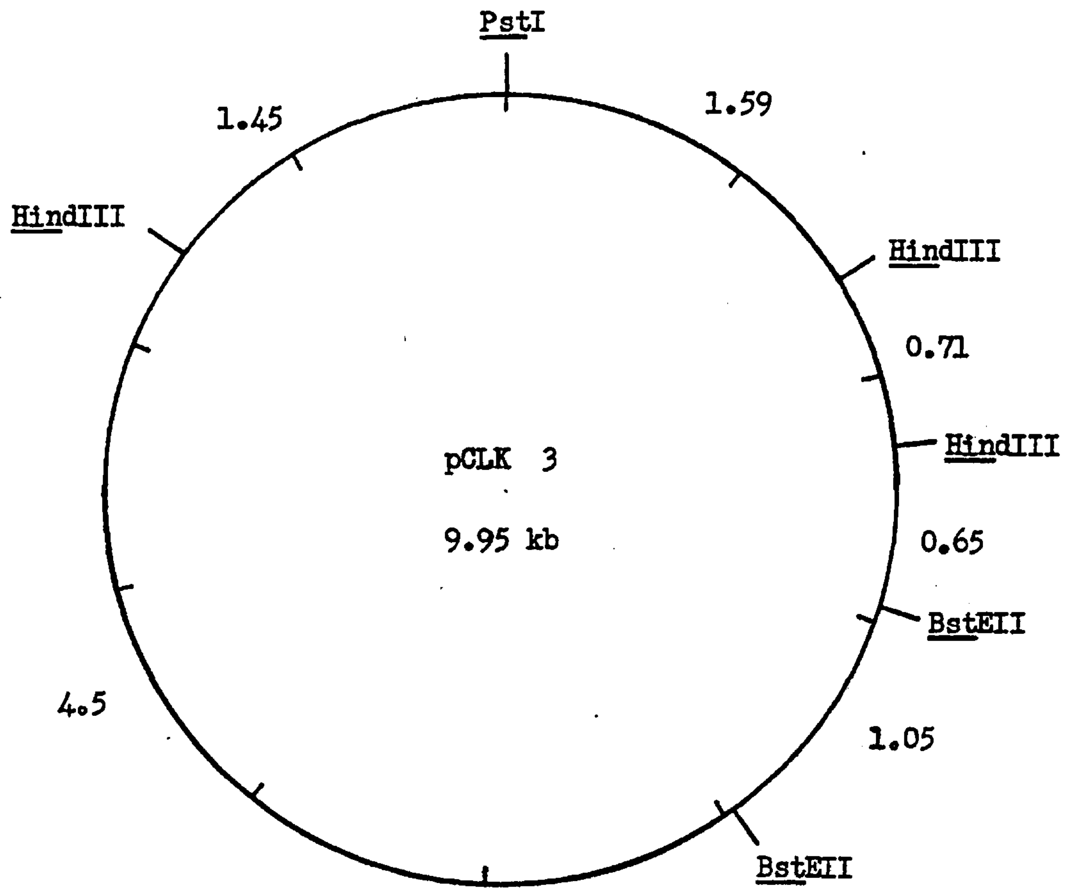


Fig. 16. The pCLK3 restriction endonuclease cleavage map.

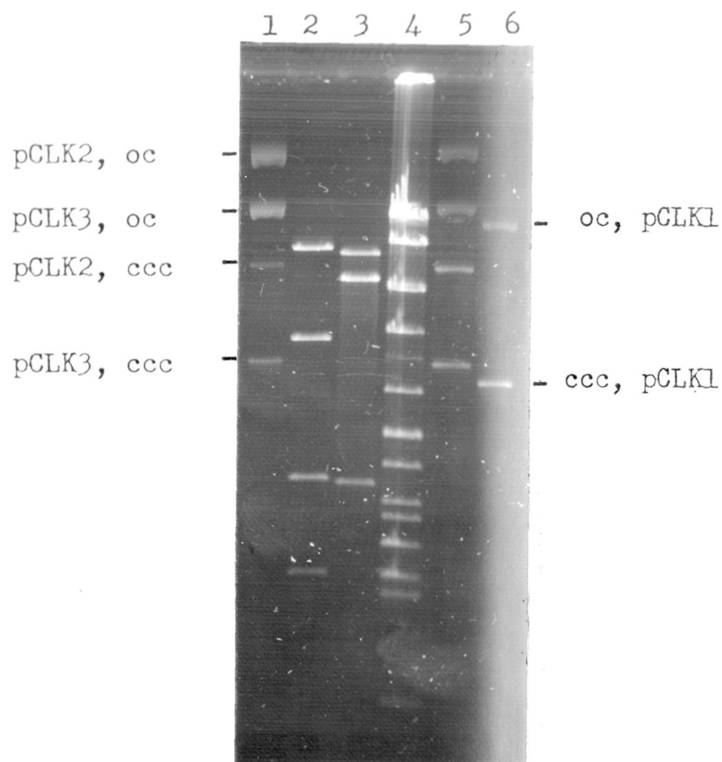


Fig. 17 (a). Agarose gel electrophoresis (0.5%) of pCLK2 and pCLK3 (plus pCLK1 for comparison). (1) & (5) Uncut pCLK2 and pCLK3 ; (6) uncut pCLK1 ; (2) pCLK2 and pCLK3 digested with HindIII and XbaI ; (3) pCLK2 and pCLK3 cleaved with PstI and XbaI ; (4) lambda DNA cleaved with HindIII plus lambda DNA digested with BstI and EcoRI.

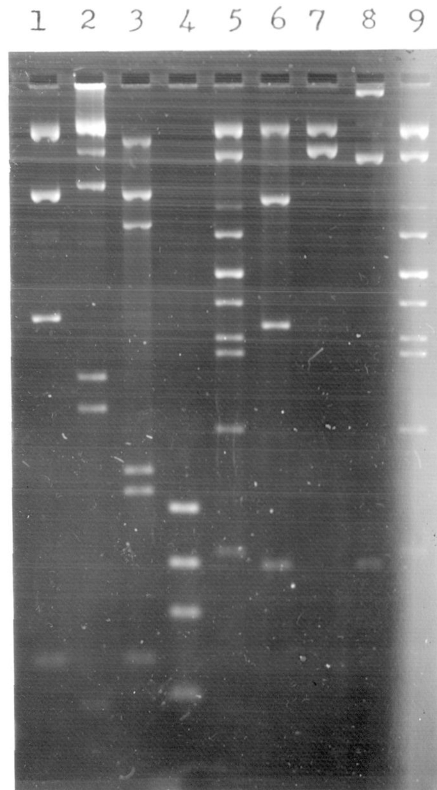


Fig. 17 (b). Agarose gel electrophoresis (1.0%) of pCLK2 and pCLK3 digested with restriction endonucleases. (2) Lambda DNA cleaved with HindIII ; (4) ØX174 digested with HaeIII ; (5) & (9) lambda DNA cleaved with BstI and EcoRI. The remaining tracks, pCLK2 and pCLK3 digested with (1) HindIII ; (3) HindIII and PstI ; (6) BstEII and Pst I ; (7) PstI ; and (8) BstEII.

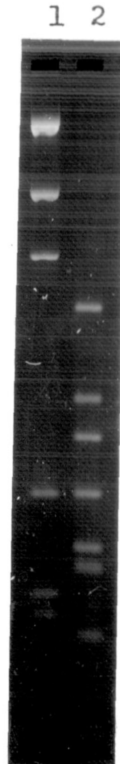


Fig. 17 (c). Agarose gel electrophoresis (1.1%) of pCLK2 and pCLK3 digested with restriction endonucleases. (1) pCLK2 and pCLK3 digested with BstEII and HindIII ; (2) ØX174 digested with HaeII plus ØX174 cleaved with HaeIII.

5.5 Discussion

The three plasmid-bearing *T. aquaticus* strains were found to be sensitive to a number of antibiotics. It was also found that the presence of plasmids in them could not be correlated with bacteriocin production. Although the strains should be tested for several other functions usually associated with plasmids in other bacterial species, these plasmids are tentatively considered as cryptic. Hishinuma *et al.* (1978) have also discovered the presence of cryptic plasmids in *T. flavus* and *T. thermophilus*. It appears that plasmids are quite common among this group of extreme thermophiles, although so far no biological function has been identified with them. In contrast to these Gram-negative extreme thermophiles, a number of the plasmids which were isolated from the Gram-positive thermophilic bacilli were shown to carry antibiotic resistance markers (Bingham *et al.*, 1979, 1980; Imanaka *et al.*, 1981). Plasmids pAB124 (Bingham *et al.*, 1979, 1980) and pTB20 (Imanaka *et al.*, 1981) were found to carry tetracycline resistance markers, while pTB19 (Imanaka *et al.*, 1981) was shown to bear both kanamycin and tetracycline resistance determinants. Perhaps a concerted screening programme should be launched to isolate antibiotic-resistant *Thermus* species from natural sources and then to detect plasmids which confer resistance to antibiotics on *Thermus*.

One plasmid, designated pCLK1, was identified in *T. aquaticus* B1, and two plasmids, designated pCLK2 and pCLK3, were detected in *T. aquaticus* B2. From the restriction endonuclease digestion patterns, the sizes of pCLK1, pCLK2 and pCLK3 were determined to be 8.76 kb, 17.24 kb and 9.95 kb respectively.

An interesting feature in the pattern of endonuclease digestions is that with few exceptions, all these three plasmids are not cleaved or sparingly cleaved by *Bcl*I (TGATCA) , *Bgl*II (AGATCT) , *Bst*EII (GGTNACC) , *Eco*RI (GAATTC) , *Hind*III (AAGCTT), *Hinf*I (GANTC) , *Hpa*I (GTTAAC) , *Kpn*I (GGTACC) , *Pst*I (CTGCAG) , *Sal*I (GTCGAC) , *Xba*I (TCTAGA) , and *Xor*II (CGATCG); and are cleaved at many sites by *Alu*I (AGCT) , *Ava*II (GG$^{A}_{T}$CC) , *Bst*I (GGATCC) , *Bgl*I (GCCNNNNNGGC) , *Eco*RII (CC$^{A}_{T}$GG) , *Hae*II (PuGCGCPy) , *Hae*III (GGCC) , *Hpa*II (CCGG) , *Sma*I (CCCGGG) , *Sst*I (GAGCTC) , *Sst*II (CCGCGG) , and *Taq*I (TCGA). These observations indicate that these three plasmids, isolated from the extremely thermophilic *T. aquaticus* strains, are very sensitive to restriction endonucleases whose recognition sequences contain a high percentage of G and C, but are less sensitive to those endonucleases which recognize sequences having a high percentage of A and T. A few exceptions were noted.

The observed pattern of sensitivity to restriction endonucleases suggests that these plasmids from the extremely ther-

mophilic _T. aquaticus_ have high GC contents like their chromosomal DNA (62 - 65 mol % GC, Pask-Hughes and Williams, 1977). This feature was also noted among the plasmids isolated from the other extreme thermophiles, _T. flavus_ and _T. thermophilus_. (Hishinuma _et al._, 1978) (Table 17). The GC contents of their chromosomal DNA were reported to be 69 - 70% (Oshima and Imahori, 1971; Saiki, Kimura and Arima, 1972). From the data in Table 17, it can be concluded that the plasmids isolated from _T. aquaticus_ are different from those isolated from _T. flavus_ and _T. thermophilus_.

A detailed restriction endonuclease cleavage map of pCLK1 showing the relative positions of the sites of nine endonucleases was constructed. It was shown to possess four single-cleavage sites (_Bgl_II, _Pst_I, _Sal_I, and _Xor_II). Restriction endonuclease maps were also constructed for pCLK2 and pCLK3, although they are not as detailed as that of pCLK1. This is due to the fact that these two plasmid species are present in the same DNA sample, limiting the restriction endonuclease digestion analyses to only certain restriction endonucleases. Plasmid pCLK2 was shown to have three single-cleavage sites (_Hind_III, _Pst_I, and _Xba_I), and pCLK3 has one single-cleavage site (_Pst_I).

A more detailed restriction endonuclease cleavage map of pCLK2 and pCLK3 could be constructed once they are separated on

TABLE 17

Number Of Restriction Endonuclease Cleavage Sites In *Thermus* Plasmids

Source	Hishinuma *et al.* (1978)			Present study		
Enzyme (seq) \ Plasmid	pTF1	pTF61 pTF62	pTT8	pCLK1	pCLK2	pCLK3
*Bst*I (GGATCC)	5	9	11	7	m	m
*Eco*RI (GAATTC)	0	0	0	0	0	0
*Hin*dIII (AAGCTT)	0	2	0	0	1	3
*Hpa*I (GTTAAC)	0	1	1	0	0	0
*Sma*I (CCCGGG)	16	12	10	m	m	m

(seq) : recognition nucleotide sequence of the restriction endonuclease

m : multiple sites (6 or more)

the basis of difference in size by rate-zonal sucrose density gradient centrifugation (El-Gewely and Helling, 1980). The individual plasmid species could then be digested by a variety of restriction endonucleases and mapped according to the technique used in mapping pCLK1. In this way, the present physical restriction endonuclease cleavage maps of pCLK2 and pCLK3 could be improved to include more sites of other restriction endonucleases.

CHAPTER SIX

CONSTRUCTION OF HYBRID DNA WITH THERMUS PLASMIDS AND THE SEARCH FOR A SURROGATE HOST

6.1 Introduction

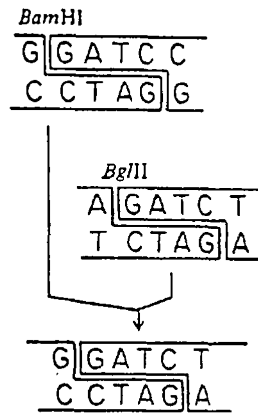
Three plasmids from *T. aquaticus* strains have now been identified and partially characterized with respect to antibiotic resistance, bacteriocin production and restriction endonuclease digestions. Although the biological functions, if any, specified by these plasmids have not yet been identified, they must contain all the functions necessary for them to exist as autonomous replicons in the extreme thermophiles. Consequently, they provide a simple system for the study of DNA replication at high temperatures. They also provide a natural source of a replication system for the *in vitro* construction of suitable plasmid cloning vectors in the extreme thermophiles. In fact, their restriction endonuclease cleavage maps suggest that there are a number of sites in which they can be linked by *in vitro* means to a selectable marker. The acquisition of a phenotypic trait would greatly facilitate studies on their transfer and their use as vectors in gene cloning in the *Thermus* species.

At the moment, there is no known transformation procedure in the *Thermus* species. The lack of a transformation system prevents the introduction of hybrid plasmid molecules into the *Thermus* species, thus making the selection of *in vitro* constructed hybrid plasmid molecules virtually impossible. This problem could be circumvented if these plasmids isolated from the Gram-negative thermophilic *Thermus* could replicate in a well-characterized surrogate host, particularly the Gram-negative mesophilic *E. coli*. If this were possible, it would mean that a shuttle or bridge vector system between *Thermus* and *E. coli* could be constructed. Such a shuttle vector system would be very useful as *E. coli* would provide a reliable and convenient host system for the selection of *in vitro* constructed hybrid plasmids containing the thermophilic plasmid replicons. Once a hybrid plasmid, containing a thermophilic replication region and preferably a selectable marker from a thermophilic source, has been established in *E. coli*, it can then be prepared in large quantities in *E. coli* and used to develop a transformation procedure in *Thermus*. With the availability of a surrogate host, it is also possible to use *in vitro* genetic labelling technique (Timmis *et al*., 1975; Niaudet and Ehrlich, 1979) to isolate the replication region of the *Thermus* plasmids in *E. coli*. It would be interesting to compare the nucleotide

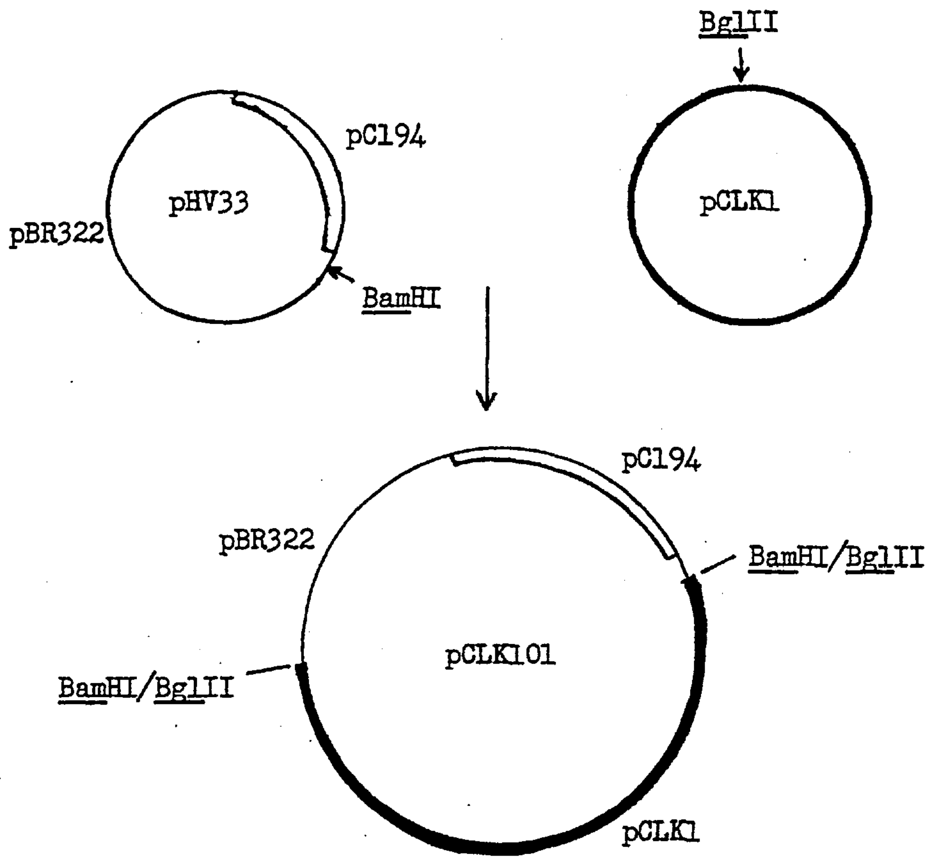
sequence in the replication origin of the extreme thermophile with that of a mesophile.

6.2 Construction of a chimeric plasmid between pCLK1 and pHV33

pHV 14 (Ehrlich, 1978) is a hybrid plasmid between *E. coli* plasmid pBR322 and *S. aureus*/ *B. subtilis* plasmid pC194, ligated at the *Hin*dIII sites of both plasmids. When it is transformed into *E. coli*, it confers resistance to ampicillin and chloramphenicol on the host. The tetracycline gene on the parental plasmid pBR322 has been suppressed by the integration of pC194 in its *Hin*dIII site (i.e. insertional inactivation). Plasmid pHV33 is a tetracycline resistant (Tc^R) revertant of pHV14 (S.D. Ehrlich, personal communication), conferring resistance to ampicillin, chloramphenicol and tetracycline on the *E. coli* host. It has a single *Bam*HI or *Bst*I site (G↓GATCC) within its tetracycline resistance gene. Plasmid pCLK1 has a single *Bgl*II site (A↓GATCT), and when it is digested by *Bgl*II, it is converted into a linear fragment having cohesive ends which are identical to those produced by *Bst*I digestion (Fig. 18). The linear fragment of pCLK1 generated by the *Bgl*II digestion can therefore be ligated to the *Bst*I generated fragment of pHV33, inactivating the tetracycline resistance gene of pHV33 by its insertion.



(a)



(b)

Fig. 18. (a) BamHI (or BstI)/ BglII fusion.

(b) Formation of pCLK101.

0.5 μg of pHV33 and 0.5 μg of pCLK1 were completely digested with BstI and BglII respectively. The BstI digested pHV33 DNA was treated with bacterial alkaline phosphatase to prevent self-ligation (Chapter Two, Section 2.26). After phenol extraction and alcohol precipitation, the two DNA species were dissolved in 50 μl of ligation buffer to a final concentration of 20 μg/ml, and ligated as described in Chapter Two (Section 2.26). The ligation mixture was used to transform E. coli strain SK1592 to ampicillin resistance. 163 Ap^R transformants were obtained on LB plates containing ampicillin. 100 of these colonies were first gridded onto LB + ampicillin plates and then replica plated onto LB + ampicillin + tetracycline plates to screen for Tc^S colonies. 84 Ap^R Tc^S transformants were identified and they were later found to be Cm^R as well. This result indicates that 84% of the transformants harbour hybrid plasmid in which a DNA insert is linked to pHV33 at its BstI site, thus inactivating the Tc^R gene.

Plasmid DNA was extracted from several of these clones and analysed with BstI. All of them generated the same fragments as the BstI digest of pCLK1, except that in the hybrid DNA, e.g. pCLK101, the fragment A of pCLK1 has become larger in size (Fig. 19), as a result of the insertion of pHV33 in the BglII site of pCLK1. This ligation result provides

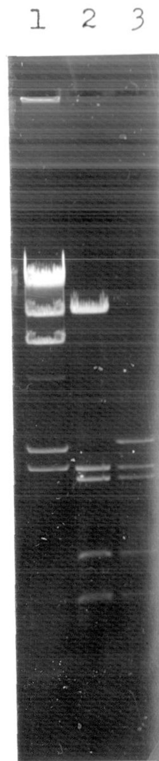


Fig. 19. Agarose gel electrophoresis (0.7%) of BstI-cleaved pCLK101 and pCLK1 plasmid DNA. (1) Reference lambda DNA digested with HindIII; (2) pCLK101 digested with BstI ; and (3) pCLK1 cleaved with BstI.

further evidence that the *BglII* site of pCLK1 lies within the fragment A of *BstI* digest of pCLK1 (Chapter Five, Section 5. 4.1, Table 13 and Fig. 13). The chimeric plasmid, pCLK101, was found to transform *E. coli* strain SK1592 to ampicillin and chloramphenicol resistance at high frequency, 1 - 1.5 x 10^5 transformants/ μg DNA, and also to retain pCLK1 stably in *E. coli* over a number of generations.

The result of this cloning experiment shows that the thermopilic plasmid, pCLK1, can be stably maintained in *E. coli* when it is stitched into an *E. coli* replicon. It is not certain whether the replication origin of pCLK1 is active in *E. coli* in this hybrid DNA since it is maintained by the pBR322 replication origin in pCLK101.

6.3 Construction of chimeric plasmids between pCLK3 and pC194 capable of replicating in *E. coli*

As mentioned in the Introduction, it is highly desirable to utilize the cryptic plasmid replicon from *Thermus* to construct a hybrid plasmid vector containing the intact replication origin of the thermophilic cryptic plasmid and a selectable genetic determinant, preferably also isolated from *Thermus*. In this way, a hybrid plasmid vector which is capable of replicating and expressing in *Thermus* will be

available for the development of a transformation system in *Thermus*.

However, currently, the main obstacle to achieving this aim is that there is no known transformation procedure to introduce the *in vitro* constructed hybrid DNA molecules back into *Thermus* for selection. To overcome this, it is absolutely essential to find a surrogate bacterial host in which the cryptic plasmid replicons can stably replicate after having been transformed into it. This surrogate host would then be able to provide a host system for the selection of a desired hybrid DNA replicon. Once such a hybrid plasmid DNA replicon, having a replication origin and a genetic determinant capable of replicating and expressing in *Thermus*, has been established in the surrogate host, it can then be prepared in large amounts and used to launch a concerted attack in the elucidation of a transformation procedure in *Thermus*.

A possible surrogate host for the Gram-negative *Thermus* plasmids is *E. coli*, which has been shown to support the replication of many plasmids from a variety of mesophilic Gram-negative bacterial species. Two methods could be used to determine whether the cryptic plasmids from *T. aquaticus* could replicate in *E. coli*. One method involves the concurrent transformation of competent *E. coli* by two sepa-

rate plasmid DNA species, one being a selectable plasmid and the other a cryptic plasmid lacking detectable phenotypic properties. The former is used as an indicator or probe to identify transformed cells that might also acquire the latter, which otherwise would be nonselectable. This indirect selection procedure permits selection of any plasmid replicon capable of being transformed into *E. coli*, and also capable of replicating in it (Kretschmer, Chang and Cohen, 1975).

The other method is the *in vitro* genetic labelling technique of Timmis *et al.* (1975) and Niaudet and Ehrlich (1979). This method involves the joining of a segment of DNA containing a replication origin which is capable of replicating in *E. coli* to another piece of DNA segment which carries a selectable marker but is not capable of autonomous replication in *E. coli*. The hybrid DNA molecule, carrying the replication origin and the selectable marker, is then transformed into *E. coli* and selected in *E. coli*.

The latter method has three advantages over the former method: 1) it involves direct selection of a replicon active in *E. coli*; 2) it involves only one replicon and therefore does not have to be concerned with the incompatibility factor; and 3) it does not only show that the replicon con-

cerned is active in E. coli, but with the right kinds of hybrid DNA molecules generated, it could also locate the DNA segment carrying the replication region of the replicon.

In this work, the latter method was preferred. Usually the segment of DNA carrying the selectable marker is excised from a plasmid which can replicate in E. coli and has therefore to be carefully isolated from any contaminating E. coli replication regions. The experiment performed here, however, used a novel approach. For this purpose, plasmids pC194, pCLK2 and pCLK3 were used.

Plasmid pC194 (Ehrlich, 1977) is a small plasmid carrying a Cm^R gene isolated from the Gram-positive S. aureus. It can transform B. subtilis to chloramphenicol resistance and is capable of autonomous replication in B. subtilis. It can also be transformed into E. coli but its chloramphenicol resistance gene is not adequately expressed to confer chloramphenicol resistance on E. coli (Goze and Ehrlich, 1980). However, when it is linked at its HindIII site to a replicon which replicates at high copy number in E. coli, such as pBR322, it confers chloramphenicol resistance on its E. coli host (e.g. pHV 14, Ehrlich, 1978; Section 6.2). It can therefore be used in isolating cryptic replicons active in E. coli.

Plasmid pCLK2 has a single cleavage site for HindIII, while pCLK3 is cleaved by HindIII into three fragments (6.2 kb, 3.05 kb, and 0.71 kb). One of the HindIII fragments from pCLK3 might contain the replication region of this cryptic plasmid, and if this replicon could replicate in *E. coli* at a reasonably high copy number, then pC194 would be able to express its chloramphenicol resistance in *E. coli* when it is linked to this cryptic replicon.

0.5 μg of pC194 and 0.5 μg of plasmid DNA (pCLK2 and pCLK3) from *T. aquaticus* B2 were completely cleaved with HindIII. After phenol extraction and alcohol precipitation, ligation was performed with the former and the latter DNA in a volume of 50 μl as described in Chapter (Section 2.26). The ligation mixture was used to transform *E. coli* strain JA221 to chloramphenicol resistance.

22 Cm^R transformants were obtained. No Cm^R transformant was observed in the control experiments in which JA221 was spread onto LB + chloramphenicol plates without any plasmid being added or after transformation with self-ligated HindIII - pC194 or self-ligated HindIII - pCLK2 and HindIII-pCLK3. The presence of Cm^R colonies after transformation with the pC194 - pCLK2 - pCLK3 ligated mixture indicates that

pC194 has been linked to a replicon which is capable of replicating in E. coli at a reasonable high copy number so as to allow it to express its chloramphenicol resistance in E. coli, like when it is linked to pBR322 (e.g. pHV 14, Section 6.2). As there were only two types of replicons involved in the ligation mixture, one from pC194 which is not effective in E. coli to express its own chloramphenicol resistance, and the other from the T. aquaticus cryptic plasmids, it indicates that the cryptic replicons from the Gram-negative Thermus plasmids can function in the Gram-negative mesophilic E. coli.

Plasmid DNA molecules were extracted from the 22 Cm^R clones by the procedure of Kado and Liu (1981) (Chapter Two, Section 2.18), and were analyzed by agarose gel electrophoresis. A representative gel is displayed in Fig. 20. Three different plasmid classes (Class I, II, and III) could be recognized which are bigger than the intact pC194. Representative plasmids of these three classes were cleaved by HindIII endonuclease, and the fragments generated were analysed by agarose gel electrophoresis (Fig. 21). The number and structures of plasmids belonging to each class are shown in Table 18.

All the plasmids isolated appeared to be hybrids between

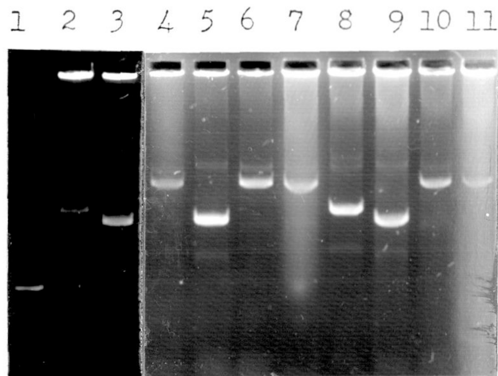


Fig. 20. Electrophoretic analysis of plasmids from Cm^R transformants of E. coli (0.7% agarose gel). (1) Reference plasmid pC194 from B. subtilis; (2) - (11) plasmids from Cm^R transformants of E. coli. (2) Class I; (3) class II ; (4) class III.

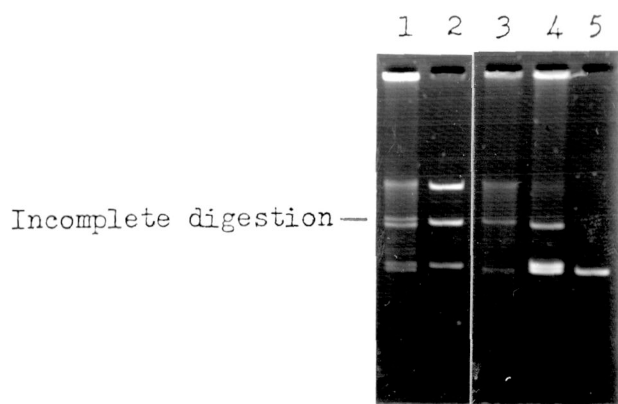


Fig. 21. Electrophoretic analysis of HindIII-cleaved plasmids. (1) pCLK301 ; (2) pCLK2 and pCLK3 ; (3) pCLK303 ; (4) pCLK302 ; and (5) pC194.

TABLE 18

Plasmid Classes Obtained Upon *in vitro* Joining Of pC194 And pCLK3

Class	No. of isolates	Representative	HindIII-released DNA fragments (M.W. kb) pC194 origin	pCLK3 origin
Parental	-	pC194	2.9	
Parental	-	pCLK3		6.2,3.05,0.71
I	2	pCLK301	2.9	6.2,3.05,0.71
II	8	pCLK302	2.9	6.2,3.05
III	12	pCLK303	2.9	6.2

pC194 and pCLK3. None of the plasmids was composed of pC194 alone, confirming the observation of Goze and Ehrlich (1980) that pC194 replicates poorly in E. coli by itself and does not transform E. coli to chloramphenicol resistance. None of the hybrid molecules was composed of pC194 and pCLK2. Perhaps the HindIII site in pCLK2 is located within its replication region. As expected, all these plasmids from the Cm^R clones contain pC194, carrying the Cm^R gene. The only segment of pCLK3 origin common to all these three classes of hybrid plasmids is the largest one with a size of 6.2 kb, suggesting that it is necessary for the autonomous replication of these hybrid plasmids in E. coli, as well as the expression of pC194 in E. coli.*

All the three hybrid plasmids, pCLK301, pCLK302 and pCLK303, were found to transform E. coli strain JA221 to chloramphenicol resistance at high frequency, about $1 \times 10^4 - 1 \times 10^5$ transformants / μg DNA.

Three conclusions can be drawn from these data. (1) Plasmid pCLK3, isolated from the extreme thermophile T. aquaticus, can replicate in E. coli at a reasonably high copy number to allow pC194 to express its Cm^R trait. This means that E. coli can be used as a surrogate host to construct hybrid plasmids involving pCLK3. These hybrid

* Other possibilities are that the 6.2 kb fragment does not contain the replication functions but acts to enhance the efficiency of replication of pC194 in E. coli, allowing the expression of its Cm^R gene, or acts to promote the expression of the Cm^R gene in pC194.

plasmids could then be used to develop a shuttle vector system between *E. coli* and *T. aquaticus*. (2) The *S. aureus/ B. subtilis* plasmid pC194, carrying the Cm^R gene, can be used to label cryptic replicons which carry a *Hin*dIII site and are active in *E. coli*. This is indeed a convenient way to test whether a foreign replicon is active in *E. coli*, because pC194 can be easily prepared in large quantities free of any contaminating *E. coli* active replication regions from a suitable strain of *B. subtilis*. (3) The replication functions of pCLK3 are contained within the largest segment released by *Hin*dIII endonuclease action.

The most important discovery in this experiment, of course, is that pCLK3 can replicate in its surrogate host, *E. coli*. Furthermore, DNA segment inserted into its *Hin*dIII sites does not interfere with its replication ability in *E. coli*.

6.4 Attempt to clone the leucine gene(s) from *T. aquaticus* in *E. coli*

One of the cryptic plasmids, pCLK3, from *T. aquaticus* B2 has been found to replicate in *E. coli*. Its replication region is contained in the largest fragment (6.2 kb) released by *Hin*dIII digestion. It is highly desirable to link pCLK3

or part of pCLK3 with its replication region to a piece of DNA from *Thermus* carrying a specific selectable genetic determinant. In this way, a plasmid vector, having both a replication region and a genetic marker which are capable of replicating and expressing in *Thermus*, can be constructed. This plasmid will then be a powerful tool in the development of a transformation system in *Thermus*.

Recently, Nagahari, Koshikawa and Sakaguchi (1980) and Tanaka, Kawano and Oshima (1981) both demonstrated that the leucine genes from *T. thermophilus* could be cloned in *E. coli*. Hybrid plasmids were constructed consisting of pBR322 and a *Hind*III fragment derived from *T. thermophilus* chromosomal DNA. The leucine genes from *T. thermophilus* were found to complement the *E. coli* *leu*B mutation *in vivo*.

As *T. thermophilus* and *T. aquaticus* were shown to be closely related to each other (Degryse *et al.*, 1978), it was thought feasible to use the same approach to isolate the leucine genes from *T. aquaticus* in *E. coli*. To achieve this, a strain of *T. aquaticus* capable of growing on a chemically defined minimal medium (Medium 162 + glucose + ammonium chloride, Chapter Two, Section 2.4) is necessary. The four *T. aquaticus* strains obtained from Dr. R.A.D. Williams were tested and among them, only one, *T. aquaticus* H, was found to be

able to grow on Medium 162 + glucose + ammonium chloride.

Total cellular DNA from T. aquaticus H, grown in 200 ml of liquid Medium 162 + glucose + ammonium chloride, was isolated by the modified method of Marmur (1961) (Chapter Two, Section 2.20), and was treated with RNase to remove the contaminating RNA molecules. It was extracted with chloroform-isoamyl alcohol (24 : 1, v/v) twice and spooled as described in Section 2.20.

0.5 μg of the purified T. aquaticus H cellular DNA and 1.5 μg pBR322 plasmid DNA were completely digested with HindIII endonuclease, heated at 70°C for 5 minutes to inactivate the HindIII endonuclease, and then phenol extracted and alcohol precipitated. The digested DNA molecules were then ligated by T4 DNA ligase at 15°C for 18 hours in 100 μl ligation buffer (Chapter Two, Section 2.26) before being used to transform E. coli strain JA221. The bulk of the transformed cells was plated onto M9 + glucose + tryptophane + ampicillin medium to select for Ap^R Leu^+ transformants, while samples were plated onto LB medium and LB + ampicillin medium to monitor the transformation frequency. The percentage of insertion in the HindIII site of pBR322 was determined by replica plating 200 Ap^R colonies onto LB + tetracycline plates. Colonies which were sensitive to tetracycline were counted

and they arose as a result of the insertion of DNA fragment into the *Hind*III site of pBR322. In a typical experiment, the average percentage of transformation was 0.85 - 1.0 x 10^{-4} , and the percentage of insertion was 12 - 15.

A small number (15) of colonies appeared on M9 + glucose + tryptophane + ampicillin plates after incubation at 37°C for 3 - 7 days. However, when plasmids were extracted from these colonies, they were found to have the same size as pBR322, and they could not transform *E. coli* strain JA221 to Ap^R and Leu^+ . These colonies were assumed to be Leu^+ revertants of *E. coli* JA221. In fact, a few of the cells of *E. coli* JA221 were found to revert to Leu^+ when plated onto M9 + glucose + tryptophane as control.

The cloning experiments were repeated with two different mass ratios of *T. aquaticus* H cellular DNA and pBR322: 1.0 μg of the former and 1.0 μg of the latter, and 1.5 μg of the former and 0.5 μg of the latter.

However, unfortunately so far I have not managed to isolate a hybrid plasmid consisting of pBR322 and *T. aquaticus* H leucine gene(s) which could transform *E. coli* strain JA221 to Ap^R and Leu^+ .

6.5 Discussion

From the experiments performed in this Chapter, a few very important conclusions can be derived.

The first conclusion of fundamental importance is that the plasmid pCLK3, isolated from *T. aquaticus* B2, can replicate in *E. coli* which therefore serves as a surrogate host to pCLK3. This is the first example of a plasmid from an extreme thermophile replicating in the mesophilic *E. coli*.

The second very important conclusion is that DNA segments can be inserted into the *Hin*dIII sites of pCLK3 without affecting its ability to replicate in *E. coli*. Its replication functions were located within the 6.2 kb fragment released by *Hin*dIII endonuclease action.

Taken together, these two findings suggest that a major obstacle to the development of a DNA cloning system in *T. aquaticus* has now been overcome, i.e. a surrogate host, *E. coli*, has been found for one of the cryptic plasmids from *T. aquaticus*. This means that *in vitro* hybrid plasmids can now be constructed between *Hin*dIII-cleaved pCLK3 and *Hin*dIII generated DNA segments carrying specific genetic determinants, and be selected in the well-characterized surrogate host, *E. coli*. The result-

ing hybrid plasmids can then be prepared in large amounts in *E. coli* and used to develop a shuttle vector system between *E. coli* and *T. aquaticus*.

Another very important conclusion is that the *S. aureus*/*B. subtilis* plasmid pC194 (Ehrlich, 1977), carrying the Cm^R determinant, can be used as a convenient selectable marker to determine whether a foreign cryptic replicon is active in *E. coli*, and at the same time to locate its replication region in *E. coli*, provided this foreign cryptic replicon carries at least a *Hin*dIII cleavage site. The main advantage of utilizing pC194 to label genetically cryptic replication region active in *E. coli* is that it can be conveniently prepared in large quantities from an appropriate *B. subtilis* strain free from any contaminating *E. coli* replicons. This eliminates the need for tedious purification, usually necessary for separating the nonreplicating segment from its own replicator (Timmis *et al*., 1975). This novel procedure used here is potentially applicable to other cryptic replicons having at least a *Hin*dIII cleavage site.

Another important finding is that *T. aquaticus* strain H is a prototrophic strain, capable of growing in a chemically defined minimal medium (Medium 162 + glucose + ammonium chloride, Degryse *et al*., 1978). This means that a strain of *T. aquati-*

cus which is amenable to biochemical and genetic studies is now available. Nutritional markers can be isolated from this strain, and auxotrophic mutants can be induced and selected.

It is unfortunate that the leucine genes of T. aquaticus H have not been isolated in E. coli. It is conceivable that a different strategy has to be employed to isolate the leucine genes of T. aquaticus H in E. coli, like using a partial HindIII-digest of the total DNA of T. aquaticus H. If this fails, then the thermophilic leucine genes should be isolated from T. thermophilus in E. coli, following the procedures as reported by Nagahari et al. (1980) and Tanaka et al. (1981).

Eventually, it is envisaged that a hybrid thermophilic replicon consisting of pCLK3 or a part of pCLK3 carrying its replication region and the leucine genes of Thermus origin would be constructed in E. coli. This hybrid plasmid replicon, which carries a replication region and a selectable nutritional marker capable of replicating and expressing in T. aquaticus, can then be prepared in large amounts in E. coli and used to develop a transformation procedure in T. aquaticus.

CHAPTER SEVEN

GENERAL DISCUSSION

The long-term aim of this project is to construct an efficient and reproducible host-vector cloning system in the Gram-negative extreme thermophile belonging to the genus Thermus. Such an alternative cloning system would allow us to investigate into a number of problems, which are both of theoretical and practical significance, like:

1) the phenomena of thermostability of macromolecules and of thermophily;

2) whether it is possible to introduce and express genes from mesophilic bacteria in thermophilic bacteria;

3) whether the products of the cloned mesophilic genes are converted to heat resistance; and

4) the mechanism of microbial evolution and adaptation at high temperature.

Furthermore, this host-vector cloning system would allow us to manipulate the metabolism of these extreme thermophiles to provide new useful industrial fermentations. It would also provide a means to amplify the production of useful macromolecules in them.

The development of a *Thermus* DNA cloning system has been hindered by the lack of suitable vector replicons and transformation/conjugation systems. At the outset of this investigation in October 1977, there was not a single report of plasmid in thermophilic bacteria, although the literature then was burgeoning with reports revealing the presence of plasmids in the mesophilic **organisms**. Only one thermophilic bacteriophage, ØYS40 (Sakaki and Oshima, 1975), was known at that time which could infect this group of extreme thermophiles. It was isolated from *T. thermophilus* HB8 and was shown to have a limited host range. It does not infect *T. aquaticus*. Although a phage vector could be developed for the cloning of DNA in *T. aquaticus*, a plasmid vector is preferred. It is easier to develop a plasmid vector than a phage vector as the former is a simpler replicon and does not need to be characterized extensively, genetically and physically, before being used as a cloning vector. In contrast, before a phage can be used as a cloning vector, a detailed genetic and physical knowledge of the phage is required, like the λ phage in *E. coli* (Murray, 1978).

Preliminary experiments were performed to investigate whether the broad host range RP4 plasmid, isolated from the mesophilic *Pseudomonas*, could be used as a cloning vector in the thermophilic *Thermus* species. As no *Thermus* strain

exhibiting the phenotypic traits of RP4 was isolated, it was decided to look for plasmids originating in *Thermus* which could be developed into cloning vectors in this group of microorganisms. Methods were developed to screen for the presence of ccc extrachromosomal DNA in *T. ruber* and a few strains of *T. aquaticus* isolated from the Icelandic hot springs (Williams, 1975; Pask-Hughes and Williams, 1977), and to isolate the pure plasmids in large quantities.

The modified method of Marmur (1961) was found to be effective in extracting the total cellular DNA from *Thermus*, and when the DNA was applied to an agarose gel electrophoresis, the presence of fast-moving ccc DNA bands was detected in three strains of *T. aquaticus*, B1, B2 and H. This was later confirmed by extracting the total cellular DNA with acid phenol (Zasloff *et al.*, 1978), which selectively removed the linear and open circular DNA molecules, leaving the ccc moiety in the aqueous phase. This method of detecting ccc DNA molecules in *T. aquaticus* is fast, simple and effective, compared to the isotopic labelling and CsCl-EtBr density centrifugation method of Hishinuma *et al.* (1978), used in the detection and isolation of plasmids from *T. flavus* and *T. thermophilus*. In fact, it will not be surprising that the procedure developed here is effective in extracting and detecting ccc DNA molecules in other microbial strains.

To prepare large quantities of pure plasmids from the plasmid-bearing _T. aquaticus_ strains, a method, incorporating features from a number of the published procedures, was developed. This involves a preliminary dilute detergent wash (Schwinghamer, 1980) to sensitize the cells to lysozyme, followed by the complete lysis of the cells using the combined action of lysozyme at 42°C and 37°C, and SDS in the presence of 1M NaCl. After lysis, the high molecular weight chromosomal DNA was selectively precipitated (Guerry _et al_., 1973) to yield a cleared lysate, which was subsequently treated with PEG 6000 in the presence of 1M NaCl to concentrate the ccc plasmid DNA (Humphreys _et al_., 1975). Finally, the enriched ccc plasmid DNA was separated from the remaining contaminating linear and open circular DNA molecules by CsCl-EtBr density equilibrium centrifugation (Radloff _et al_., 1967). Using this procedure, 0.2 - 0.4 mg of plasmids was isolated from 1,000 ml (about 8.5 - 9.0 gm wet weight) of _T. aquaticus_ strains B1 and B2, comparable to the yield of those plasmids whose copy numbers per cell were bewteen 5 - 9, e.g. pAB118A, pAB118B and pAB124 (Bingham, 1980). Again, this procedure is relatively easy compared to the isotopic labelling technique used by Hishinuma _et al_. (1978) in the isolation of plasmids from _T. flavus_ and _T. thermophilus_. An obervation made during the isolation of plasmid from _T. aquaticus_ strain B1 was that sometimes the

plasmid yield was negligible, indicating that the plasmid in T. aquaticus B1 is dispensable and is lost in some of the colonies.

These three plasmid-harbouring strains of T. aquaticus were examined with respect to certain characteristics commonly associated with the presence of plasmids in other microorganisms. They were found to be sensitive to a variety of antibiotics, like all the other Thermus species known (Brock and Freeze, 1969; Williams, 1975; Pask-Hughes and Williams, 1977; Hishinuma et al., 1978). They were also shown to be non-producers of bacteriocin, like the plasmid-bearing strains of T. flavus and T. thermophilus (Hishinuma et al., 1978). Tentatively, the plasmids isolated from these T. aquaticus strains are considered to be cryptic, just like the plasmids isolated from T. flavus and T. thermophilus strains (Hishinuma et al., 1978).

The plasmids from T. aquaticus B1 and B2 were characterized by restriction endonuclease mapping. T. aquaticus B1 was shown to harbour one species of plasmid, designated pCLK1, and T. aquaticus B2 was found to harbour two species of plasmids, designated pCLK2 and pCLK3 respectively. The restriction endonuclease analyses showed that the sizes of pCLK1, pCLK2 and pCLK3 are 8.76 kb, 17.24 kb and 9.95 kb respectively.

All these three plasmids share a common pattern of sensitivity to restriction endonucleases. With few exceptions, they are generally not cleaved or sparingly cleaved by restriction endonucleases which recognize sequences having a high percentage of A and T, e.g. *Bcl*I (TGATCA) , *Bgl*II (AGATCT) , *Eco*RI (GAATTC) , *Hind*III (AAGCTT) , *Hpa*I (GTTAAC) and *Xba*I (TCTAGA) (all these hexanucleotide recognition sequences have 2A and 2T), and also by some endonucleases whose recognition sequences contain low A and T, and high G and C , e.g. *Bst*EII (GGTNACC) , *Kpn*I (GGTACC) , *Pst*I (CTGCAG) , *Sal*I (GTCGAC) and *Xor*II (CGATCG). On the other hand, they are cleaved at multiple sites by restriction endocucleases whose recognitiin sequences contain a high percentage of G and C, e.g. *Ava*II (GG$^{A}_{T}$CC) , *Bgl*I (GCCNNNNNGGC) , *Eco*RII (CC$^{A}_{T}$GG) , *Hae*III (GGCC) , *Hpa*II (CCGG) , *Sma*I (CCCGGG) , *Sst*I (GAGCTC) , and *Sst*II (CCGCGG).

This unique feature was also noted among the cryptic plasmids isolated from *T. flavus* and *T. thermophilus* (Hishinuma *et al*., 1978) (Table 17, Chapter Five), suggesting that the plasmids from these extreme thermophiles have high GC contents like their chromosomal DNA (*T. aquaticus*, 62 - 65% GC, Pask-Hughes and Williams, 1977 ; *T. flavus* and *T. thermophilus*, 69 - 70% GC, Oshima and Imahori, 1971; Saiki *et al*., 1972). This high GC content reflects the thermophilic origin of these DNA molecules. In contrast, plasmids of mesophilic origin (e.g.

pBR322, from E. coli, Sutcliffe, 1979) or moderately thermophilic origin (e.g. pAB124, from a thermophilic Bacillus, Bingham et al., 1979; Bingham, 1980), are rarely cleaved by SmaI (CCCGGG) and SstII (CCGCGG) , reflecting their lower GC contents (E. coli, 50.0% GC ; B. stearothermophilus, 46.7% GC, Marmur, 1960) compared to the extreme thermophiles.

Restriction endonuclease maps of pCLK1, pCLK2 and pCLK3 were constructed. Plasmid pCLK1 was shown to possess four single-cleavage sites (BglII, PstI, SalI, and XorII); pCLK2 has three single-cleavage sites (HindIII, PstI, and XbaI), and pCLK3 was shown to have one single-cleavage site (PstI).

The physical endonuclease cleavage maps of pCLK2 and pCLK3 are not as detailed as that of pCLK1, but could be improved further once they are separated from each other by rate-zonal sucrose density gradient centrifugation on the basis of difference in size (El-Gewely and Helling, 1980).

Three plasmids have now been isolated from T. aquaticus and were partially characterized with respect to antibiotic-resistance, bacteriocin production and restriction endonuclease digestions. They are potential vectors for the cloning of DNA in Thermus since their sizes are not too big and they contain a number of single-cleavage sites for some

restriction endonucleases. However, at present they cannot be exploited for this purpose yet, mainly because they do not fulfil a very important requirement, which is to carry an easily selectable genetic marker. These cryptic plasmids must, nevertheless, contain all the functions necessary for them to exist as autonomous replicons in the extreme thermophiles. They are therefore a suitable natural source of a replication system for the construction of cloning vectors in the extreme thermophiles, just like the cryptic miniplasmid P15A in _E. coli_ (Chang and Cohen, 1978) and the cryptic plasmid pLS28 in _B. subtilis_ (Tanaka and Sakaguchi, 1978), which were used to construct a series of special-purpose plasmid cloning vehicles. It is desirable to link by _in vitro_ means a selectable genetic marker, preferably of _Thermus_ origin, to one of these thermophilic cryptic plasmid replicons in order to construct a readily discernible cloning vector whose replication origin and genetic determinant are capable of replicating and expressing in the extremely thermophilic _Thermus_ strains. This hybrid plasmid replicon would then be a very powerful tool in the development of a host-vector cloning system in _Thermus_.

To achieve this, a transformation procedure is needed to introduce the _in vitro_ constructed hybrid plasmids into _Thermus_, so as to allow the desired chimeric plasmid vector to

express itself and be selected. However, since there is no known transformation procedure in *Thermus*, it is impossible to select the desired *in vitro* constructed hybrid vector in *Thermus* strains. This problem could be circumvented if it were possible to introduce these thermophilic plasmids into a surrogate bacterial host strain in which they could be stably propagated. In this way, a desirable hybrid plasmid constructed by *in vitro* means could be selected in the surrogate host, prepared in large amounts, and then used to develop a transformation procedure in *Thermus*, particularly *T. aquaticus*. Such a shuttle or bridge vector system between two different hosts would be very useful, especially if one of the hosts was *E. coli*.

Plasmid pCLK1 was cleaved with *Bgl*II restriction endonuclease and linked to the *Bam*HI (or *Bst*I) site of pBR322 in the hybrid pHV33 (which is pBR322 and pC194 , a plasmid isolated from the Gram-positive *S. aureus*/*B. subtilis*, linked at the *Hind*III sites of the two plasmids). The resulting chimeric plasmid, pCLK101, was shown to be stably maintained in *E. coli*, conferring ampicillin and chloramphenicol resistance to the host. However, it is not certain whether the replication origin of pCLK1 in pCLK101 is active in *E. coli* since it is maintained by the pBR322 replication origin in pCLK101.

To ascertain whether one of the cryptic plasmids from T. aquaticus could replicate in E. coli and at the same time to locate its replication region, the in vitro genetic labelling technique of Timmis et al.(1975) and Niaudet and Ehrlich (1979) was used. The basic idea is to link a segment of DNA containing a replication origin which is capable of autonomous replication in E. coli to another piece of DNA which carries a selectable marker but is not capable of replicating in E. coli. In this way the replication origin linked to a selectable marker is identified. Usually, the segment of DNA carrying the selectable marker is excised from a plasmid which can replicate in E. coli and has therefore to be carefully isolated free from any contaminating E. coli replication origins. The experiment performed in this study used a novel approach.

The plasmid pC194 , isolated from the Gram-positive S. aureus/B. subtilis, was used as the selectable marker as it carries a Cm^R gene. Although it can be transformed into E. coli, it does not replicate effectively by itself in E. coli and does not confer chloramphenicol resistance to E. coli, unless it is linked to a replicon which replicates at a reasonably high copy number in E. coli, e.g. pBR322 (Goze and Ehrlich, 1980). In this way, it can be used to show whether a foreign replicon is active in E. coli, and at the same time to

locate the replication origin of the foreign replicon. The advantage of using pC194 to label genetically cryptic replication regions active in *E. coli* is that it can be prepared in large amounts from an appropriate *B. subtilis* strain free from any contaminating *E. coli* replicons.

In this investigation, the replication region in question comes from a non-*E. coli* source, i.e. from *T. aquaticus*, and the experiment performed was to investigate whether this foreign replication region can replicate in *E. coli*, and if it does, whether this region can be identified and isolated. *In vitro* constructed hybrid DNA molecules among *Hin*dIII-cleaved pC194 and *Hin*dIII-cleaved pCLK2 and *Hin*dIII-cleaved pCLK3 were used to transform *E. coli* to chloramphenicol resistance. Cm^R clones were obtained in this case, but not in experiments when self-ligated *Hin*dIII-cleaved pC194 and self-ligated *Hin*dIII-cleaved pCLK2 and pCLK3 were used to transform *E. coli* respectively. These results show that pC194 has been linked to a replication region active in *E. coli* so as to allow it to express its Cm^R trait in *E. coli*; and the replication regions of the thermophilic cryptic plasmids from *T. aquaticus* are active in *E. coli*.

Plasmids were isolated from the Cm^R clones and were all found to be larger than the intact plasmid pC194. Based on

their mobilities in the agarose gel, three classes of hybrid DNA molecules were identified. Representative plasmids of these three classes were cleaved by HindIII endonuclease, and the fragments generated were analysed by agarose gel electrophoresis. All of them were found to contain two common DNA segments : the 2.9 kb pC194 and the 6.2 kb fragment of HindIII-cleaved pCLK3. This suggests that the 6.2 kb fragment from pCLK3 released by the HindIII endonuclease action contains the replication functions of pCLK3. No plasmid contained pC194 alone or pC194 ligated to pCLK2. It could be possible that the HindIII site in pCLK2 lies within its replication region.

Three very important conclusions are derived from the previous experiment. (1) Plasmid pCLK3 from *T. aquaticus* B2 can replicate in *E. coli*, which therefore serves as a surrogate host to pCLK3. This is in fact the first example of a plasmid from the extreme thermophile *T. aquaticus* replicating in the mesophilic *E. coli*. (2) DNA segment can be inserted into the HindIII sites of pCLK3 without interferring with its ability to replicate in *E. coli*. This means that hybrid plasmids can be constructed in *E. coli* between HindIII-cleaved pCLK3 and HindIII generated DNA fragments carrying specific genetic determinants. (3) The Gram-positive *S. aureus*/ *B.*

subtilis plasmid pC194, carrying the Cm^R gene, can be used as a convenient selectable marker to test whether a foreign cryptic replicon is active in E. coli and to locate its replication region in E. coli, provided the cryptic replicon carries at least a HindIII cleavage site.

An important barrier to the development of a DNA-cloning system in T. aquaticus has now been overcome, i.e. a surrogate host, E. coli, has been found for one of the cryptic plasmids from T. aquaticus. Fortunately, this surrogate host is well characterized, genetically and physiologically. This means that desirable hybrid plasmids containing the thermophilic replication region can be constructed in vitro using E. coli as the host for selection and amplification.

Next, it is desirable to isolate a segment of DNA from T. aquaticus which carries a selectable genetic determinant, such as the leucine genes. This segment can then be subsequently linked to the thermophilic replication region to create a hybrid replicon which has the ability to replicate and express in the extreme thermophiles. Such a plasmid replicon would be a powerful tool in the elucidation of a transformation procedure in T. aquaticus. The leucine genes of T. aquaticus were chosen as the candidates for the selectable genetic determinant because it was recently reported by

Nagahari *et al.* (1980) and Tanaka *et al.* (1981) that the leucine genes of *T. thermophilus* had been cloned in *E. coli*.

Among the few *T. aquaticus* strains available, *T. aquaticus* strain H was found to grow satisfactorily in a chemically defined minimal medium (Medium 162 + glucose + ammonium chloride, Degryse *et al.*, 1978). It must therefore be prototrophic and possess all the functions necessary for it to survive in a minimal medium. It also means that this strain is amenable to biochemical and genetic studies as auxotrophic mutants can be induced and selected.

Following the procedures of Nagahari *et al.* (1980) and Tanaka *et al.* (1981), purified total cellular DNA was prepared from *T. aquaticus* H and was digested to completion with *Hind*III restriction endonuclease. Attempts were then made to clone the *T. aquaticus* H leucine genes with these *Hind*III generated DNA fragments into the *Hind*III site of pBR322 in *E. coli* strain JA221, a *leu* auxotroph. However, unfortunately, no hybrid plasmid has yet been isolated which can transform *E. coli* strain JA221 to Ap^R and Leu^+ phenotype.

It is conceivable that a different strategy has to be employed to isolate the leucine genes of *T. aquaticus* H in *E. coli*, failing which the thermophilic leucine genes should

be isolated from T. thermophilus in E. coli as reported by Nagahari et al. (1980) and Tanaka et al. (1981). Eventually, it is envisaged that a thermophilic replicon consisting of pCLK3 or a part of pCLK3 carrying its replication region and the leucine genes of Thermus origin, would be constructed in E. coli and used to develop a transformation procedure in T. aquaticus.

In conclusion, the work presented in this dissertation has demonstrated that it is possible to develop a DNA-cloning system in T. aquaticus. Many of the prerequisites essential to the development of such a host-vector system, which were not available at the outset of this project, are now available: methods to screen for and to isolate plasmids from T. aquaticus; thermophilic replicons in the form of cryptic plasmids; endonuclease cleavage maps of three of the cryptic plasmids; a prototrophic strain of T. aquaticus, strain H, for the isolation of desired genes and the induction of auxotrophic mutants for biochemical and genetic analyses; a convenient method of labelling genetically cryptic replicons in E. coli; a thermophilic plasmid (pCLK3) which can replicate in a surrogate host, E. coli; and a well-characterized surrogate host, E. coli, for the development of a shuttle/bridge vector system between T. aquaticus and E. coli.

This work has laid the foundation for further work to elucidate a transformation procedure in T. aquaticus, once a hybrid replicon which can replicate and express in T. aquaticus has been established in E. coli. It has also shown that the replication region of a thermophilic plasmid can be isolated. It would be of fundamental interest to compare the nucleotide sequence in this replication region of an extreme thermophile with that of a mesophile, e.g. E. coli.

This work, together with that of Hishinuma et al. (1978), have demonstrated that plasmids are quite ubiquitous in the genus Thermus, although so far no biological function has been identified with them. In contrast to these Gram-negative extreme thermophiles, a number of the plasmids which were isolated from the Gram-positive moderately thermophilic bacilli were shown to carry antibiotic resistance markers (Bingham et al., 1979, 1980; Imanaka et al., 1981). Therefore, I feel that parallel to the efforts to construct in vitro thermophilic plasmid vectors having a selectable marker active in T. aquaticus, a more vigorous screening of Thermus strains from natural sources should be performed to find indigenous plasmids that carry a discernible genetic determinant or selectable markers. Ultimately, I am confident that a host-vector cloning system will be developed in this group of extreme thermophiles, belonging to the genus Thermus.

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