'Cloning the enterotoxin gene from *Clostridium perfringens* type A'

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Dedicated to my family, in particular my Mother and Father, Stella and James Goodman, my husband Henryk, daughter Christine, and my Mother and Father-in-law, Stanislawa and Michal Iwanejko.

It is necessary to recognise that with respect to unity and coherence, mythical explanation carries one much further than scientific explanation. For science, does not, as its primary objective, seek a complete and definitive explanation of the Universe. . . It satisfies itself with partial and conditional responses. Whether they be magical, mythical or religous, the other systems of explanation include everything. They are applied to all domains. They answer all questions. They account for the origin, for the present and even for the evolution of the universe.

FRANÇOIS JACOB

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ABSTRACT

A *C. perfringens* type A genomic library was constructed in *E. coli* by banking overlapping 6-10 kbp *Hin*d III fragments of chromosomal DNA from the enterotoxin (CPE) positive strain NCTC 8239 into the pUC derived vector pHG165. The library was screened by colony hybridization with a degenerate 26 bp oligonucleotide probe, derived from the amino acid sequence CPE_{9-17}. A complex mixture of plasmid DNA was isolated from the only hybridization positive clone. A second round of screening picked out a single plasmid, with an apparently altered copy number, pLW1, that carried the CPE gene, *cpe*, on a 6.8 kbp insert.

A sequence deduced primer strategy for direct plasmid sequencing was initiated using a primer deduced in a similar manner to the 26 bp probe, obviating the need for prior mapping and subcloning of the insert. The amino acid sequence for the conceptual gene product of the single open reading frame differed only slightly from the known CPE sequence but lacked the C-terminal residues. The biased *cpe* codon usage reflected the low %G+C content of the DNA. The %G+C content was even lower in the upstream region and possessed properties characteristic of bent DNA. The region 5' to the ATG translational start codon contained a Shine-Dalgarno sequence and several sequences with significant homology to the putative transcriptional control regions for the tetanus toxin gene. The N-terminal coding region contained a direct repeat of an upstream sequence that shared considerable homologies with the crossover point in site 1 of the Tn*3 res* region.

Southern blot analyses of chromosomal and plasmid DNAs from several isolates indicated that the majority of strains were *cpe*$^-$. The chromosomal location and architecture of *cpe* appeared identical in all *cpe*$^+$ strains.

A second copy, pLW2, of the 5' end of *cpe*, on a 4.5 kbp *Pst* I/*Eco* RI restriction fragment, was cloned during one of many unsuccessful attempts to clone the 3' end. A separate re-cloning experiment isolated several different clones that contained the 0.6 kbp *Hin*d III located ≅ 2.5 kbp 5' to the ATG codon of both cloned copies of *cpe* but none of them carried the CPE gene. The fragment was used as a DNA probe to show that it was present in high copy number in some strains of *C. perfringens* but completely absent from others.

An hypothesis describing the possible involvement of a mobile genetic element in *C. perfringens* enterotoxin production offers explanations for the cloning of a complex mixture of plasmids, the apparent alteration in plasmid copy number, the identification of putative DNA crossover points, the failure to clone the 3' end of *cpe* and the isolation of a novel DNA fragment.

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To my friends and colleagues Simon, Phil, Brian and Tania I extend an affectionate word of thanks for helping me to keep my sense of humour.

It is impossible to convey the debt I owe to all of family and friends who over the years have extended to me their unquestioning love, support (financial and moral) and encouragement, just saying thank-you seems inadequate.

PREFACE

'Cloning the enterotoxin gene from *Clostridium perfringens* type A' was an AFRC funded research studentship. The studentship comprised three years scientific research work, conducted in the Food Microbiology Laboratory in the Department of Applied Biochemistry and Food Science, University of Nottingham, under the supervision of Dr. Gordon S. A. B. Stewart. That work is reported here. The thesis is divided into three main sections, Introduction, Experiments and Discussion.

The Introduction starts with a description of the genus *Clostridium* emphasizing the diversity of morphological and biochemical characteristics shown by its members. A detailed description of the study organism, *C. perfringens*, is provided by Chapter 2. The different toxin types and the diseases associated with them are described with particular reference to the subject of this thesis, *C. perfringens* type A food poisoning. The chemical and biological details of the proteinaceous agent of the illness, the 35.4 kDa enterotoxin molecule, are also presented. Chapter 3 sets out the reasons for trying to clone *cpe*, the enterotoxin gene. The initial objectives of the project are presented concluding with a résumé of accomplishments. Also described are some of the experimental strategies used e.g. DNA sequencing, employed during the course of the investigations. The provision of this chapter enables the reader to focus on the scientific aims and discussions of the studies, detailed in the following chapters, without the distraction of lengthy technical explanations of each experimental procedure. Protocols and details of materials are given in the appendices.

Preliminary work carried out by M. Routledge, a post-graduate student initially assigned to the project, is clearly identified in the first of six "Experiments" chapters. This section details the cloning and DNA sequencing of the N-terminal coding region and upstream sequences of the enterotoxin gene, *cpe*, along with the attempts to clone the C-terminal coding sequences and 3' flanking DNA. Chapters 6 and 7 are concerned with the experiments which addressed the questions regarding the distribution and plasmid or chromosomal location of *cpe*. The last "Experiments" chapter describes the unexpected cloning of an unusual DNA fragment.

The Discussion brings together all the information obtained during the course of the studentship. The contributions they make to the understanding of the biological processes involved in *C. perfringens* type A food poisoning are discussed along with suggestions for future research.

INTRODUCTION
CHAPTER 1
THE CLOSTRIDIA

1. 1. Circumscription of the Genus *Clostridium*

In the late 19th century the anaerobic, spore-forming bacilli were separated from the genus *Bacillus* and re-classified as *Clostridium* (L. *Clostridium,* spindle). The *Clostridium* were defined as Gram-positive rods that were obligate anaerobes and had central or subterminal, heat resistant, spores which swelled the cell. The type species being *C. butyricum* [*Prazmowski*, 1880]. Over one hundred years later the principally morphological description of the genus, which in 1880 was due to a lack of knowledge at any other level, has changed little. The generally accepted definition in *Bergeys Manual Of Systematic Bacteriology*. Volume 2. [*Cato et al.*, 1986] identifies the *Clostridium* as anaerobic or micro-aerophilic, spore-forming rods that do not form spores in the presence of air, are usually Gram-positive, and do not carry out a dissimilatory sulfate reduction.

1. 2. Morphological and Chemical Characteristics

1. 2. 1. Gross Morphology

There are few morphological constraints and therefore the clostridia are a highly pleomorphic group of bacteria. The cells of most species are large (0.5 - 2.0 µm in diameter and up to 30 µm in length) rods. The exceptions being *C. coccoides* (oval), *C. cocleatum* (semi-circular) and *C. spiroforme* (spiral). The rods may be thick or thin, straight or curved and have blunt, pointed or rounded ends. Filamentous forms are not uncommon. Most species are motile with peritrichous flagella but there are some encapsulated non-motile species e.g. *C. perfringens.*

An organism can only be included in the genus if it possesses a Gram-positive (single-layered) cell wall. In a few species e.g. *C. magnum* this is only identifiable using electron microscopy. Some species e.g. *C. aminovalericum* are Gram-variable, varying with culture age and conditions.

Although all clostridia are spore-formers there is considerable variation in spore morphology and frequency of sporulation. In all cases the oxygen, heat and alcohol resistant spores are only produced under anaerobic conditions. The motile or non-motile spores may be terminal, subterminal, central or all three and they usually, but not always, swell the cell. *C. oceanicum* has two

spores per cell. The majority of clostridia will sporulate if a general sporulation media is used e.g. chopped meat agar [*Holdeman et al.*, 1977] and incubated at 5 - 10°C below the optimum growth temperature. Species with specialised metabolisms usually only sporulate in more precise medias e.g. *C. polysaccharolyticum* requires cellobiose and rumen fluid before it will sporulate. Sometimes spores cannot be demonstrated visually e.g. *C. ramosum*, in which case spore formation is assumed to have occurred if the organism survives alcohol or heat (80°C or 70°C for 10 minutes) treatment. Unfavourable treatments e.g. subculturing and/or heat treatment are often employed to increase spore:vegetative cell ratios in species with low sporulation frequencies e.g. *C. perfringens*.

The ever increasing size (there are over 80 described *Clostridium* species) and diversity of the genus has incited several authors [*Chester*, 1901; *Weinberg et al.*, 1937; *Handuroy et al.*, 1937; *Prévot*, 1938 and 1940] to suggest division of the clostridia into smaller genera. The organisms would be categorised according to cell shape and spore position etc.. The proposals have so far been rejected on the grounds that a morphological classification system for these bacteria would not help the process of identification.

1. 2. 2. Chemical Analysis

1. 2. 2. 1. Walls and Membranes

Cell wall components are potentially useful aids to bacterial identification [*Cummins & Harris*, 1956]. The type of peptidoglycan and its constituent amino acids, especially diaminopimelic acid, typify the genera whilst the species are distinguished by the sugar components of the wall. Clostridial peptidoglycans are usually of the *meso*-diaminopimelic acid direct-linked type. Most *Clostridium* species contain an assortment of sugars in their cell walls e.g. *C. acetobutylicum* has glucose, galactose, rhamnose, and mannose. The genus can be divide into those species which have both glucose and galactose e.g. *C. beijerinckii* and those which have glucose but do not have galactose e.g. *C. butyricum*. Teichoic acids, polymeric phosphodiesters, have been isolated from several species of *Clostridium*, this is not unusual as the walls of most Gram-positive bacteria contain other polymers in addition to the basic peptidoglycan.

C. thermohydrosulfuricum, a thermophilic species, has been shown to have a proteinaceous S layer surrounding the cell wall [*Sleytr & Glauert*, 1976]. The hexamerically patterned surface of this species was revealed using freeze-etching and electron microscopy.

There is very little known about clostridial cytoplasmic membranes. The

protein components are assumed to have similar functions to those in other Gram-positive bacteria i.e. import and export of vital materials. The membrane bound redox carriers used in the transfer of electrons from oxygen to substrates with lower oxidation levels to enable storage of free energy were excluded from this assumption. It was thought that clostridia could not use such a system because they are obligate anaerobes and energy storage is unnecessary as they derive all that they require from fermentation. Indications to the contrary came with the discovery that some acetogenic species could use either CO_2 + H or simply CO as their sole supply of carbon and energy [*Wiegel et al*, 1981; *Kerby & Zeikus*, 1983]. For acetate to be synthesised, during carbon monoxide utilisation, it seemed likely that ATP had to be generated. Investigations revealed the presence of a variety of electron transport proteins e.g. cytochrome *b* in *C. thermaceticum* [*Rogers*, 1986] which could be involved in electron transport coupled phosphorylation.

There is only slightly more information available on the lipid content of clostridia membranes. In all bacteria the precise lipid composition varies with culture conditions, especially pH and temperature. In general the major class of lipids are the phospholipids with long chain fatty acids making up the remainder. The fatty acids of Gram-positive cytoplasmic membranes are frequently branched but rarely unsaturated, whereas Gram-negatives use a diverse mixture of fatty-acids, both saturated and unsaturated, with branched-chains often entirely absent. There are also Gram-associated differences in membrane phospholipid content. Phosphatidylethanolamine is the phospholipid in Gram-negatives whilst the Gram-positives utilise many different types of phospholipid, although they never use phosphatidylcholine (lecithin) the major lipid component of eukaryotic membranes. In clostridia the phospholipids are plasmologens i.e. the aldehyde form e.g. *C. butyricum* contains the plasmologens *N*-methylethanolamine, ethanolamine and phosphotidylglycerol.

1. 2. 2. 2. Proteins

Species identification by means of polyacrylamide gel electrophoresis (PAGE) of cellular proteins has been examined [*Cato et al.*, 1982; *Bom et al.*, 1986] and found to be suitable for the separation of two known species, even closely related species such as *C. bifermentans* and *C. sordellii*. However a definitive species identification system for the clostridia using PAGE is not a feasible proposition because cellular protein composition varies with subculturing, culture age and growth conditions e.g. some toxin production is reliant on the possession of mobile genetic elements [*Crowther & Baird Parker*, 1983; *Strom et al.*, 1984] which may be lost during successive subculturing.

1. 2. 2. 3. Fatty Acids

Some of the fatty acids associated with the genus were discussed earlier (**1. 2. 2. 1. Walls and Membranes**). Analytical gas liquid chromatography, at the species level, has found that the specific metabolic pathways of the clostridia are reflected in their fatty acid composition [*Elsden et al.*, 1980]. It may eventually be possible to incorporate this phenomenon into a bacterial identification system.

1. 2. 2. 4. Nucleic Acids

The overall G+C composition of clostridial DNA is very low (G+C = 22mol% → 55 mol%, averaging at about 28 mol%). DNA isolated from *C. perfringens* [*Iwanejko et al.*, 1989] was assumed to be methylated. The DNA was not cut by *Sau* 3A and *Bam* HI, restriction enzymes which do not recognise sequences which contain 5-methylcytosine, however it was cleaved by *Mbo* I, an isoschizomer of *Sau* 3A, which is not inhibited by 5-methylcytosine residues but is sensitive to methylated adenine residues. A number of bacteriophage are associated with the clostridia (see below) so it is likely that DNA restriction/modification systems are present within the genus.

1. 3. Growth and Metabolism

1. 3. 1. Overview of Habitat and Growth Requirements

The ubiquitous clostridia are found most commonly in anaerobic habitats such as the mammalian intestinal tract or in environments with low oxygen tension such as soil, marine sediments and decaying organic matter. Diversity within the genus *Clostridium* is not limited to morphological characteristics. The members vary in their oxygen tolerances and growth requirements i.e. preferred substrate, temperature and pH.. The clostridia have a fermentative metabolism which allows them to obtain their ATP via substrate level phosphorylation. The discovery of electron-transport type proteins in some clostridia (**1. 1. 2. 2. Walls and Membranes**) is evidence that some species, perhaps not all, have the means to generate a proton-motive force which could be utilised for additional ATP production. If an electron-transport chain does exist it is unlikely that O_2 is used as the terminal electron acceptor, as is frequently the case in other systems, as there are very few clostridial species able grow in the presence of molecular oxygen. *C. carnis*, *C. histolyticum*, *C. durum* and *C. tertium* are known as oxygen tolerant species because they are able to grow in air but they do not survive for long in these conditions nor are they able to sporulate [*van Gylswyk & van der Toorn*, 1987]. Defined as anaerobic or microaerophilic most species are sensitive to oxygen, the highly

sensitive species *C. haemolyticum* only grows when the oxygen tension (pO_2) is less than 0.5% and only survives a few minutes in air. The majority of species are less fastidious and fall between the two extremes e.g. *C. novyi* type A will grow with a pO_2 up to 3.0% and *C. butyricum* can survive (but not grow) for hours in air.

Clostridia may be aerointolerant because they lack the ability to protect themselves from the detrimental effects molecular oxygen and its metabolites e.g. the highly reactive free radical, O_2^{-}. , the superoxide anion produced by partial reduction of O_2 (see **Figure 1.**). Toxicity is believed to be due to the interference of normal cell metabolism and the chemical alteration of vital macromolecules. However the enzyme superoxide dismutase (SOD), which is thought to afford this protection to aerobic organisms by catalysing the dismutation of the super oxide anion to hydrogen peroxide (H_2O_2), has been detected in several clostridial species [*Gregory et al.*, 1978]. There is no evidence to indicate the general absence of SOD from the remaining species, although *C. tertium*, a micro-aerophilic species, is completely devoid of SOD, so it is unlikely that SOD on its own is the only prerequisite for aerotolerance. It is not known whether clostridia possess catalase, the companion enzyme to SOD, which catalyses the conversion of the potentially lethal hydrogen peroxide to water and oxygen

Optimal growth of most anaerobes occurs in media with low redox-potential (E_h), this may underlie the reason why aeration of a clostridial culture is often inhibitory. The presence of oxygen in a medium is likely to cause an increase in its E_h, by virtue of the fact that it is an avid electron acceptor i.e. potent oxidant. Although there are some psychrophilic and thermophilic *Clostridium*, for most species growth is optimal within the narrow pH and temperature ranges (pH 6.5-7.0; 30-37 °C); since E_h is affected by changes in these factors, as exemplified by the modified redox values*, E'_0 , for the redox-couple NADH/ NAD^+, * E'_0 = -320 mV but E_h = -230m at pH 4.0, then a requirement for a particular value could be what imposes the restrictions on these parameters. The idea that these factors i.e. pH and temperature, are not by themselves growth limiting is substantiated by the observation that for some species the pH and temperature required for optimum growth varies with the substrate provided.

* E'_0 = standard oxidation-reduction potential (pH 7.0, 25°C).

Figure 1.
Possible Routes to Oxygen Sensitivity

1.Products of Reduction Reactions.

	Comments
$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$	Complete reduction produces the ubiquitous and harmless water molecule.
$O_2 + 2H^+ + 4e^- \longrightarrow H_2O_2$	A two electron partial reduction of O_2 results in hydrogen peroxide formation which is lethal at high concentrations.
$O_2 + e^- \longrightarrow O_2^{-}.$	A single electron reduction produces the superoxide anion, a highly reactive *free radical.

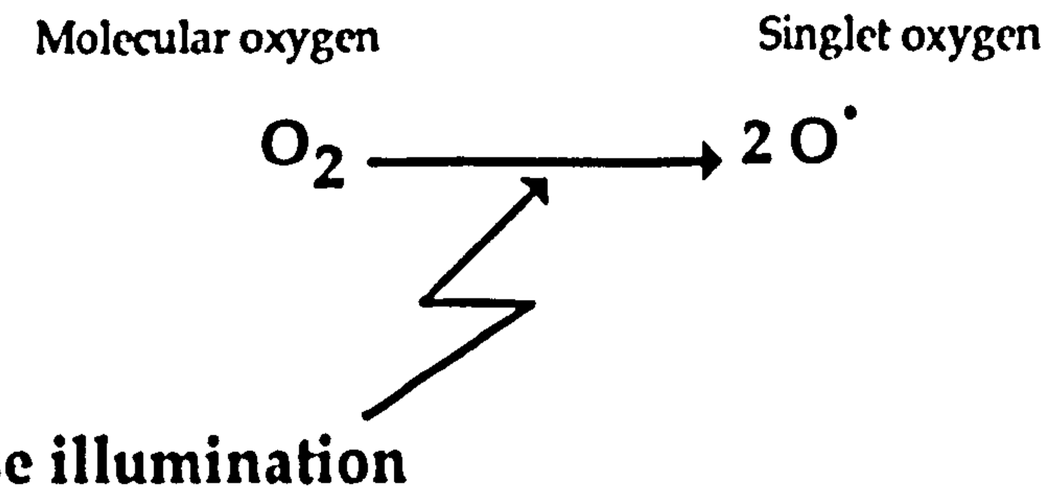
2. The toxicity of the superoxide anion and hydrogen peroxide may be because they are the precursors to the hydroxyl free radical (HO.) which is also a product of ionizing radiation.

Haber-Weiss Reaction

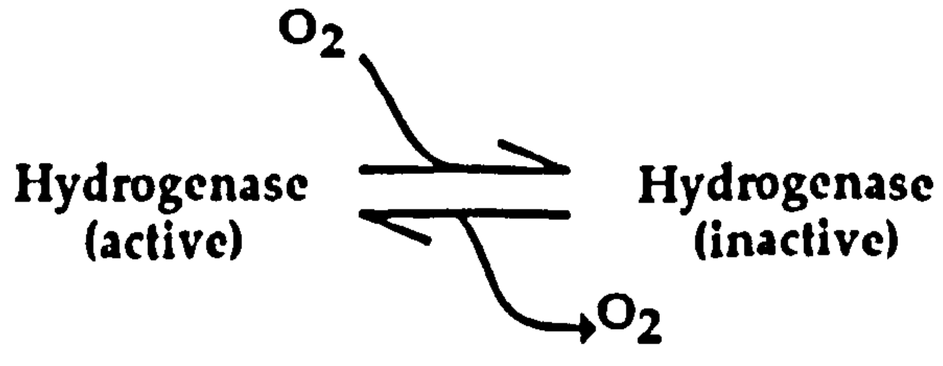
$$O_2^{-}. + H_2O_2 \longrightarrow OH^- + HO.$$

Fe(III)

3. Photodynamic Cell Death



4. Interaction with enzymes and/or co-factors e.g. *Desulfovibrio* [*Odom & Peck*, 1984]



5. Oxygen as a Potent Oxidant.

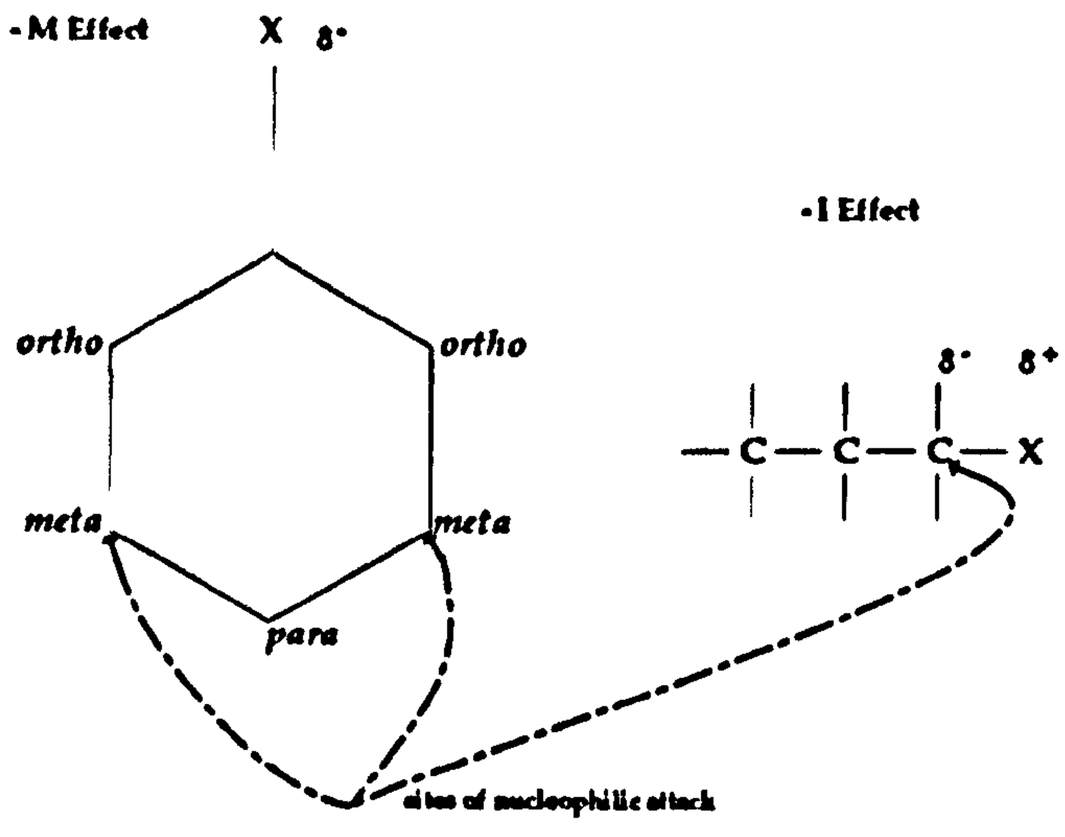
I. Affect on redox-potential (E_h) : -oxygen is an avid electron acceptor so aeration of a medium raises its E_h.

II. Depletion of NADH.

$$NADH + H^+ + \tfrac{1}{2} O_2 \rightleftharpoons NAD^+ + H_2O$$

NADH oxidase

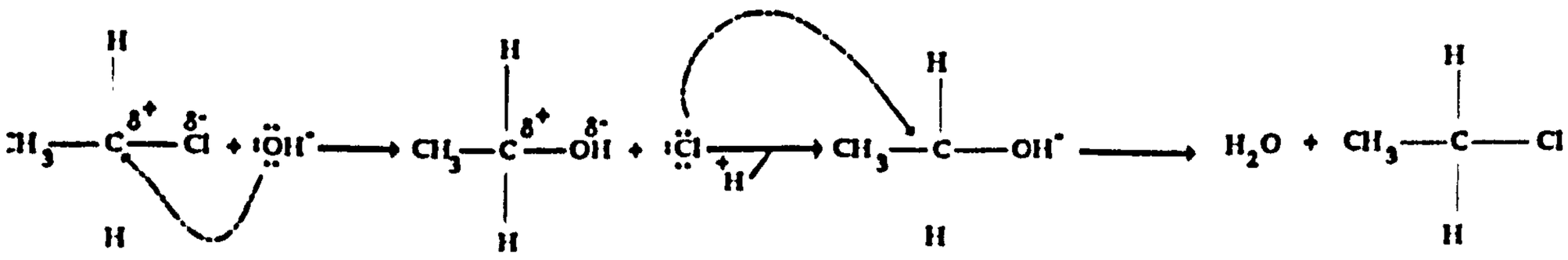
*Note:



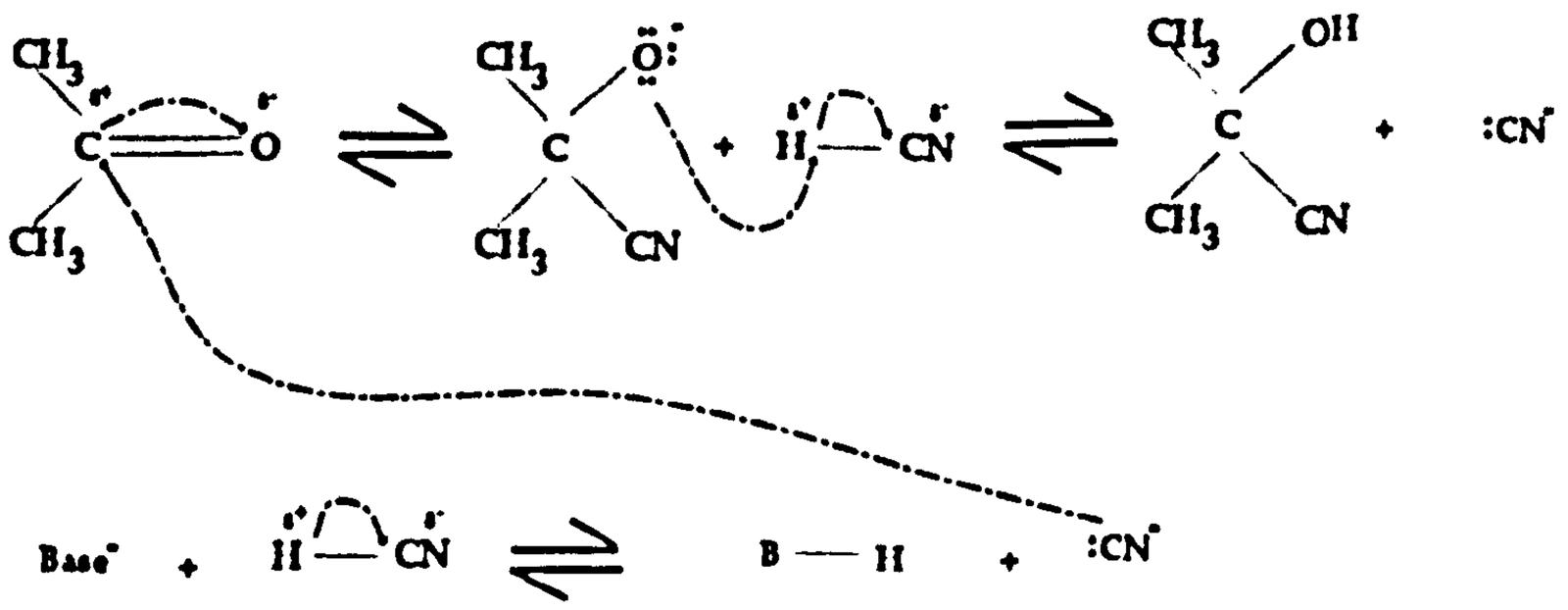
Free radicals are highly reactive because they have unpaired electrons (lone pairs) possession of which makes molecules nucleophilic i.e. they attack sites of low electron density. These types of sites exist in organic molecules when one part of the system is made electron deficient by an negative inductive (-I) or mesomeric (-M) effect of an attached moiety. These systems are very common in biological macromolecules.

A nucleophilic substitution reaction occurs when the system is a saturated carbon chain with an electronegative partner. Such attachment groups draw electrons from the system i.e. have a -I effect, most groups e.g. OH are of this type. The only common +I agents are alkyl groups. Nucleophilic addition reactions occur with unsaturated systems, such as aromatic rings, which have low electron densities. This situation results when the attached group has an electronegative element, such as a double bond, which withdraws electrons from the system i.e. has a -M effect. Both nucleophilic addition and substitution reactions dramatically alter the characteristics of the 'attacked molecule', frequently producing another highly reactive species which goes on to do further damage.

Nucleophilic Substitution Reaction



Nucleophilic Addition Reaction



1. 3. 2. Fermentation Processes

The clostridia utilize the Embden-Meyerhof-Parnas pathway [*Thaur et al.*, 1977] and a modified Entner-Doudoroff pathway [*Andreesen & Gottschalk*, 1969] for the catabolism of sugars and sugar acids to ethanol, acetate and/or butyrate. Other fermentation products e.g. acetone, butanol and isopropanol are dependent on species specific enzymes, substrate and culture conditions, also some species switch their metabolic processes under stressed conditions resulting in in changed products of fermentation [*Andreesen et al.*, 1989].

Clostridial fermentations are an important commodity because they have the potential to provide fuels and chemicals from renewable biomass such as starch [*Ng et al.*, 1983]. The feasibility of large scale fermentations for the commercial production of chemicals has been demonstrated [*Ng et al.*, 1983]. It is hoped that increased understanding of clostridial biochemistry and genetics will lead to the development of genetically engineered organisms which will be more effective fermenters and provide greater yields than the wild type strains [*Wood*, 1981]

1. 4. Clostridial Genetics

1. 4. 1. Genomic Analysis

A variety of clostridial genes encoding, both toxic determinants and metabolic enzymes, have been cloned and analysed. The %G+C content of clostridial DNA was found to be extremely low 22-55 (T_m) even the thermophile *C. thermocellum*, which has an optimum growth temperature of 60-64°C, has a %G+C of only 38-39 (T_m) [*Cato et al.*, 1986]. The non coding region of clostridial genes has an even lower G+C content which is thought to be a feature common to clostridial promoter/operator regions [*Leslie et al.*, 1989]. The lack of available data concerning transcription initiation sites has hampered attempts to define the general characteristics of clostridial promoter regions [*Young et al.*, 1989a & b]. Characteristic factor-independent transcription-terminator sequences i.e. regions of dyad symmetry preceding, or including a stretch of T residues, have been found to correspond to the mRNA 3' termini for the *C. pasteurianum* ferredoxin [*Graves & Rabinowitz*, 1986] and the *C. thermocellum celA* [*Béguin et al.*, 1986] genes. The 6-13 nucleotide separation of the putative Shine-Dalgarno sequences and translation codon, usually AUG, [*Young et al.*, 1989a & b] is similar to the spacing found in *E. coli* ribosome binding sites. The codon usage however was significantly different to that of *E. coli* genes and reflected the high A-T content of clostridial DNA.

1. 4. 2. Extrachromosomal Elements; Plasmids, Transposons and Bacteriophages

Many clostridial extrachromosomal genetic elements have been identified. The majority of the plasmids remain cryptic although several have been shown to code for drug resistance and bacteriocins [*Abraham & Rood*, 1985a & b; *Garnier & Cole*, 1988a]. Plasmid mediated and plasmid free, intraspecific and interspecific, conjugal gene transfer has been reported for the clostridia [Review: *Young et al.*, 1989b]. The conjugative plasmid, *C. perfringens* pIP401, is also able to mobilize the transfer of the non-conjugative plasmid pIP402 [*Bréfort et al.*, 1977]. pIP401 carries a 6.2 kbp transposon, Tn*4451* [*Abraham & Rood*, 1987 and 1988] encoding the chloramphenicol resistance determinant, which can excise precisely leaving a deleted version of the plasmid. Tn*4451* appears to be related to the Tn*3* family of transposons. pPI404, the most extensively characterised clostridial plasmid, encodes several UV inducible genes and a *res* gene which encodes a protein with significant homology to the transposon encoded resolvases [*Garnier et al.*, 1987 and 1988].

The first isolation of a clostridial bacteriophage was from *C. tetani* in 1934 [*Cowles*, 1934] since then phage have been isolated from pathogenic species e.g. *C. botulinum* [*Vinet & Fedetle*, 1968] and important industrial species e.g. *C. madisoni* [*Ogata & Hongo*, 1979] where they interfere with the fermentation processes. Some phages are the toxic determinants of pathogenic clostridia. They either carry the structural genes for the toxins, exemplified by the conversion of non toxigenic *C. botulinum* to toxigenic forms [*Ecklund et al.*, 1971; *Inoue & Iida*, 1971], or they influence the expression of host encoded toxin genes as occurs in *C. novyi* Types A and B [*Ecklund et al.*, 1976].

1. 5. The Pathogenic Clostridia

About fourteen of the eighty three species decribed in *Bergey's Manual of Systematic Bacteriology*. Volume 2, cause illness or disease in humans or other animals. A further six species are associated with disease [*Smith & Williams*, 1984]. *C. botulinum*, *C. tetani*, *C. perfringens*, *C. difficile* and *C. septicum* are the species most harmful to the health of human beings. The neurotoxins of *C. botulinum* and *C. tetani* being amongst the most dangerous toxins so far discovered. Yet surprisingly the anticholinergic effect of the *C. botulinum* neurotoxin type A is being exploited as a therapeutic agent. It is being used to alleviate involuntary spasmodic muscle contractions in conditions such as focal dystonias [*Scott*, 1980] and to cause selective weakening of certain overactive muscles [*Lee et al.*, 1988; *Elston*, 1988].

All the biologically active, antigenic factors produced by the pathogenic

clostridia are termed toxins even if they have not been assigned to a role in disease pathology [Review: *Hatheway*, 1990]. A number of toxins are common to different *Clostridium* species but the spectrum of toxins produced by an isolate can be used for taxonomic purposes.

1. 7. Phylogenetic and Evolutionary Considerations

The gėnus clostridia has been subdivided into homology groups on the basis of DNA-ribosomal RNA (rRNA) competition studies. The groupings correlated well with earlier DNA-DNA hybridization studies [*Johnson*, 1970] but they do not correlate well with the phenotypic groupings used for identification e.g. spore location and cell wall structure. Although termed homology groups the intragroup variation for some groups is almost as great as the inter group variation. 16S rRNA cataloguing, described by Cato & Stackebandt [1989], of 120 Gram-positive eubacterial species from 33 genera demonstrated that the large interspecies distances were due to the phylogenetic heterogeneity of the genus. The placing of *Clostridium* species on many of the sublines of the *Clostridium/Bacillus* subdivision is indicative of an ancient ancestory [*Stackebandt*, 1988].

CHAPTER 2
CLOSTRIDIUM PERFRINGENS

2. 1. Circumscription of the Species

2. 1. 1. Historical Perspective

The first complete circumscription of the organism was provided by Welch and Nuttall [1892] when they described *Bacillus aerogenes capsulatus*, a bacillus isolated from a cadaver. The organism was later classified as *C. perfringens* toxin type A with the lamb dysentery bacillus, *C. paladus* and *C. ovitoxicum*, closely related organisms only varying from the original isolate in their toxin production, being designated by Wilson toxin types B, C and D respectively [*Glenney et. al., 1932*]. Type E, the agent of enterotoxaemia in calves [*Bosworth*, 1943] and type F from human enterotoxaemia were added later although the type F classification has since been discarded due to its basic similarity to type C [*Stern et al.*, 1964].

2. 1. 2. Isolation and Identification

C. perfringens type A, is the most widely distributed pathogenic bacterium [*Smith & Williams*, 1984]. It has been isolated from soil, water, milk, dust sewage and the intestinal canal of man and animals. It has even been isolated from soil samples in Antarctica [*Miwa*, 1975]. Toxin types B, C, D and E appear to be obligate parasites, surviving only in the intestinal tracts of animals and man [*Smith & Williams* 1984].

The clostridia are fairly strict anaerobes with an optimum growth temperature of 45°C and sporulation temperature of 37°C. Under optimum conditions they have a generation time of about 8 minutes although one strain was recorded to have a doubling time of 7.1 minutes at 41°C [cited in, *Craven*, 1980]. Limited growth accompanied by rapid cell death occurs at temperatures above 50°C and vegetative cells are unable to survive temperatures above 60°C in laboratory media [*Brown & Twedt*, 1972].The atrichous, non-motile cells are straight, parallel sided rods (≅ 4-8 μm × 0.8-1.0 μm) with truncated or slightly rounded ends (although they are sometimes shorter and thinner and filamentous forms are not uncommon) that occur either singularly or in pairs. The variation in cell morphology increases with culture age. Sporulation is rare in culture but spores are commonly found in their natural environment. The spores are large, oval, central or subterminal and frequently distend the cell.

The cell walls contain LL-DAP. Galactose, glucose and rhamnose are amongst the common cell wall sugars that may be present [*Cummins & Johnson*, 1971].

Most strains are encapsulated but the precise composition of the largely polysaccharide capsules varies amongst the strains [Review; *Smith*, 1975].
Acid gas production results from the fermentation of glucose, maltose, lactose, sucrose and occasionally salicin but not from mannitol. Some strains also ferment inulin and types A, C and D ferment glycerol.
The mol% G+C of the DNA is 24-27 (T_m) [*Cummins & Johnson*, 1971].

2. 1. 3. Toxins and Bacteriocins

The five types of *C. perfringens* produce a spectrum of exoproteins with demonstrable or potential pathogenic biological activity. Classification of strains into types A-E is according to the excretion of four major lethal toxins (**Table 2.2.**) as other morphological or biochemical methods (Chapter 1) are unable to reliably distinguish between the types. The guinea pig skin erythema method of toxin typing involves the subdermal injection of toxins and specific antisera raised in rabbits. Each *C. perfringens* type has a characteristic toxin neutralization pattern obtained by using various combinations of toxin-antitoxin [*Smith & Williams*, 1984]. Usually isolates are not usually typed as type A is the only one commonly associated with disease in man. Types B, D and E are not known to be human pathogens but type C is known to cause enteritis necroticans, a rare but severe form of food poisoning in man (**Table 2.1.**). The the β toxin of type C strains was identified as the main aetiological agent of Darmbrand, a necrotizing haemmorhagic jejunitis that occurred after the consumption of contaminated tinned meat in germany 1947 [*Hobbs*, 1979; *Walker*, 1985]. Type C enteritis is very distressing and is also associated with a high mortality rate, particularly when the victims are young Papua New Guineans. People living in the highlands of New Guinea become afflicted with the disease, known as "pig-bel", after the ritual eating of pig meat. The severity of the illness is thought to be exacerbated by a reduced ability to neutralize the toxin due the low gut protease activity of the victims. This is believed to be due to the combined effects of their low protein diet and the protease inhibitory properties of sweet potatoes their staple food [*Murrel et al.*, 1966].

Although the toxigenic type of an isolate is not required for diagnosis, confirmation of an outbreak of *C. perfringens* food poisoning does require determination of relatedness between isolates from and between the victims and the suspect food. This is achieved by serotyping for about 69% of all isolates [*Stringer et al.*, 1980]. The procedure is limited to the major reference laboratories because of the lack of commercially available antisera. Only 17 serotypes are currently on offer [Denka Sieken Co.] which is useless when account is taken of the 31% of isolates which remained non typable even using

antiseras pooled from various countries [*Stringer et al.*, 1980].

Strains of *C. perfringens* can be typed according to patterns of bacteriocin sensitivity and production [*Scott & Mahony*, 1982]. Most strains are sensitive to enterococcal bacteriocins but only about one third of strains examined have been identified as bacteriocin producers [*Scott & Mahony*, 1982]. A bacteriocin role for the type A enterotoxin was indicated when it was found to have spheroplast-forming properties which were blocked by anti-CPE gamma globulins [*Torres-Anjel & Rieman*, 1976]. Evidence in support of this comes from the observation that a higher proportion of food poisoning isolates are bacteriocinogenic compared to isolates from human and animal infections, the environment and food [*Watson et al.*, 1982].

An early investigation of *C. perfringens* bacteriocins noted that some bacteriocin production was UV inducible [*Tubylewicz*, 1966a and b]. More recent investigations have located a transcriptionally controlled gene, *bcn*, for the 95 kDa , UV inducible bacteriocin BCN5 on the conjugal plasmid pIP404 [*Garnier & Cole*, 1988c]. Its superficial similarity to the SOS controlled expression of the colicin genes of *E. coli* [*Pugsley*, 1984], which are also on conjugally transferrable plasmids, was noted.

Figure 2. 1.

Some of the Diseases of Associated With the *C. perfringens* Toxin Types A-E

Toxin Type	Diseases
A	Gas gangrene; food poisoning; infectious diarrhoea; enterotoxemia of lambs and other animals.
B	Lamb dysentery; enterotoxemia of farm animals.
C	Darmbrand; pig-bel; 'struck' in sheep; enterotoxemia of some domesticated animals.
D	Enterotoxemia of sheep; pulpy kidney in lambs.
E	Enterotoxemia in calves; lamb dysentery.

Figure 2. 2.

Distribution and Characteristics of the Toxins, Including the Enterotoxin, of *C. perfringens*

Type	Major Lethal Toxins				Minor Toxins								Nuramidase (sialidase)	Other
	a	b	e	i	g	d	h	t	k	l	m	n		Enterotoxin
A	+	-	-	-	-	-	±	±	+	-	±	±	+	+
B	+	+	+	-	±	±	-	+	+	+	+	+	+	NK
C	+	+	-	-	±	±	-	+	+	-	±	+	+	+
D	+	-	+	-	-	-	-	+	+	+	±	±	+	+
E	+	-	-	+	-	-	-	+	+	+	±	±	+	NK

Major Toxins

Alpha	Beta	Epsilon	Iota
Lecithinase (phospholipase C)	Necrotizing	Permease; trypsin activatable	Dermonecrotic
? leucocytolysin	Heat & trypsin labile	protoxin 34.75 kDa	Binary(ι-a+ι-b)
pI 5.4	28 kDa		ADP ribosylatin
43 kDa	pI 5.6		pI 5.2 & 4.2
			47.5 & 71.5 kDa

Minor Toxins

Gamma	Delta	Eta	Theta	Kappa	Lambda	Mu	Nu
?	Haemolysin 42 kDa pI 9.1	?	Heamolysin (O_2 labile) Consists of four components 59-62 kDa pI 6.1; 6.3; 6.5 & 6.8 cloned	Collagenase gelatinase	Protease	Hyaluronidase	Dnase

Neuramidase *N*-acetylneuramic acid glyohydrolase. Several different enzymes.

Enterotoxin

Cytotoxic-activity increased by trypsin and chymotrypsination [*Granum et al.*, 1981; *Granum & Richardson*, 1991]

Single polypeptide; 80% β-sheet and 20% random coil [*Granum & Harbitz*, 1985]

Heat stable to 53°C [*Granum & Skjelkvåle*, 1977]

35.4 kDa

pI 4.3

2. 1. 4. Plasmids, Transposons and Bacteriophages.

A number of extrachromosomal genetic elements, mainly cryptic plasmids, of varying sizes have been isolated from many strains of *C. perfringens*. Plasmid profiling became a potentially viable alternative typing method when it was revealed that only 23% of food poisoning isolates did not have demonstrable plasmid profiles [*Mahony et al.*, 1987] compared to the 31% of non-serotypable [*Stringer et al.*, 1980]. Also, a second plasmid profiling study revealed that a 6.2 kbp plasmid, equivalent in size to Tn*4451* and Tn*4452*, was present in the majority of plasmid bearing food poisoning isolates (71%) compared to only 19% of non food poisoning associated isolates [*Phillips Jones et al.*, 1989].

The plasmid mediated phenotypic characteristics that have so far been determined include the bacteriocin production discussed in the previous section and antibiotic resistance (**Table 2.3.**). The well characterised chloramphenicol resistance determinant of the conjugal plasmids pIP401 and pJIR27, were found to reside on 6.2 kbp transposons [*Abraham & Rood*, 1987 and 1988], Tn*4451* and Tn*4452* respectively, the only transposon yet discovered in *C. perfringens*. These elements excised precisely from recombinant plasmids used to transform a *recA* strain of *E. coli*.

The complete nucleotide sequence for the 10.2 kbp bacteriocinogenic plasmid, pIP404, has been elucidated and functions have been tentatively assigned to 6 of the 10 open reading frames [*Garnier & Cole*, 1988a; *Garnier et al.*, 1987]. The replication and copy number control functions of pIP404 have also been examined [*Garnier & Cole*, 1988b]. Essential features of pIP404 replication are the *rep* protein and an "origin-like" region composed of repeated sequences. PA1, a powerful promoter controlling the production of a 150 nucleotide RNA molecule was detected within the "origin-like" region. The sequence of the RNA molecule, RNA 1, was complementary to a part of the Rep protein mRNA which encoded a helix-turn-helix motif similar to those found in DNA binding proteins. Priming of DNA synthesis and/or an anti-sense RNA based mechanism for controlling the rate of initiation of plasmid replication and whence copy number were among the suggested roles for RNA 1. A second protein Cop was found to be needed for the maintenance of plasmid copy number.

The plasmid, pIP401 (Tc^r Cm^r), which carries the transposable element Tn*4451*, is able to mobilize other plasmids and mediate the transfer of chromosomal markers. Mobilization of the 63 kbp, non-conjugative plasmid pIP402 (Em^r Cc^r) was assumed to have occurred when the phenotype of CP720, the resultant strain from an *in vivo* mating experiment using axenic mice and a donor strain containing pIP401 and pIP402, was Tc^R Cm^R and Em^R

Cc^R. The transfer could not achieved during conventional plate mating experiments [*Bréfort et al.*, 1977 and 1978]. A separate study of pIP401 found it to have a 23 kbp region with significant homology to pCW3 [*Abraham & Rood*, 1985] which led to the hypothesis that a pCW3-core exists in all the Tc^r plasmids of *C. perfringens*.

The physical characteristics of several bacteriophages of *C. perfringens* have been described [*Mahony et al.*, 1987] but there appears to have been very little research in to their physiological attributes despite the assumed involvement phages have in the pathogenicity of other *Clostridium* species. *C. perfringens* type A food poisoning is associated with spore formation (section 2.3.) and an investigation of the temperate phage s9 found that it was associated with sporulation kinetics and heat resistance [*Stewart & Johnson.* 1977]. Only 0.2% of strains cured of the prophage produced heat resistant (80°C 15 minutes) spores compared to the 50% produced by the lysogenised strain. The cured strain also took six hours longer than the infected strain to produce refractile spores. Also, the chromosomal attachment (att) sites for the lysogenic phages, ϕ29 and ϕ59, have recently been physically mapped by Southern blot analysis (Chapter 3) of large restriction enzyme fragments separated by pulsed field gel electrophoresis [*Canard & Cole*, 1989]. The att site for ϕ29 mapped close the *nanH* gene which codes for a sialidase, a possible virulence factor.

Table 2. 3.

***C. perfringens* Plasmids** [*Young et al.* 1989]

Plasmid (kbp)	Properties Carried	*C. perfringens* Strain
pJU121 (3.3)	cryptic	12502
pJU122 (3.9)	cryptic	12502
pJU124 (38.8)	Tc^R	12502
pCW3 (47)	Tc^R; conjugative	11268
pIP401 (54)	Tc^R, Cm^R; conjugative	CP590
pIP402 (63)	Em^R, Cl^R; mobilizable	CP590
pIP404 (10.2)	BCN5 production; mobilizable	BP6K-N5
pJIR25 (52)	Tc^R, Cm^R; conjugative	CP600
pJIR27 (50)	Tc^R, Cm^R; conjugative	1329
pHB101 (3.1)	caseinase production	3626B

2. 2. *C. perfringens* Type A Food poisoning

The link between *C. perfringens* and food poisoning was not established until the early 1940s [*McClung*, 1945] when the species was the candidate organism isolated from several food poisoning outbreaks. The association was confirmed by using the isolates to reproduce the illness in human volunteers and laboratory animals [*McClung*, 1945].

The usual presenting symptoms of the illness are diarrhoea, severe abdominal pains and nausea but occasionally vomiting, shivering and pyrexia are observed. The illness is rarely fatal but deaths of elderly or otherwise debilitated victims have been recorded [*Sutton & Hobbs*, 1965]. The clinical manifestations occur 8-24 hours after the ingestion of large numbers of vegetative cells (10^6-10^7 cells g^{-1} food). The duration of the illness is 12-24 hours after the onset of the symptoms [*Hobbs*, 1969].

Outbreaks are most commonly associated with red meats and poultry dishes served in large establishments. The fastidious growth requirements of *C. perfringens* are well provided for under these conditions. The meats contain all the essential amino acids, simple peptides and vitamins needed to sustain growth, whilst the food preparation regimes in large establishments frequently involve the cooking of large amounts of food which are then held at a temperature suitable for the growth of *C. perfringens*, sometimes well in advance of serving. A survey of outbreaks during the five year period 1972-76, by the Center for Disease Control, found that most (95%) involved improper food holding conditions and a significant proportion (35%) were associated with inadequate cooking procedures [*Craven*, 1980]. Cooking procedures are inadequate if they fail to destroy both vegetative cells and spores because the heat shock activates spore germination [*Craven*, 1980]. It was believed that food poisoning strains possessed heat resistant spores but strains that lack heat resistant spores have been reportedly responsible for some outbreaks. It was postulated that the original observation was an artifact that arose partly as a result of the germination of heat damaged spores on the isolation media [*Labbe*, 1989].

Confirmation of a *C. perfringens* food poisoning outbreak is provided by viable counts in excess of 10^5 from the suspect food; the detection of large numbers of cells in the faeces of the victims; isolation of the same serotype from ill individuals but different to those obtained from healthy controls; presence of identical serotypes in the incriminated food and the faeces of afflicted persons. There are many problems associated with these confirmatory tests. The ubiquity of the organism means that its presence alone is insufficient to confirmatory evidence of an outbreak for two reasons. Firstly,

it is easy for food to become contaminate after the event and secondly, *C. perfringens* is almost always isolated from faecal samples, often in large numbers, from healthy people [*Stringer et al.*, 1985]. The process is further limited by the unreliability of the serotyping used to confirm relatedness between different isolates. This complication arises as a result of the significant number of naturally occurring isolates which spontaneously lose their antigenic capsule against which the sera are raised [*Sutton*, 1969; *Hughes et al.*, 1976; *Stringer et al.*, 1980].

The true incidence of the *C. perfringens* food borne disease is not known because of the under reporting due to the relative mildness and short duration of the illness compounded by the inability to confirm suspected outbreaks. Even without accounting for this *C. perfringens* is still responsible for a high proportion of food poisoning incidents. In the United States it is the third most important bacterium, in the United Kingdom it is second only to *Salmonella* infections [*Labbe*, 1991] and in Norway it is the primary cause of bacterial food poisoning [*Gondrosen et al.*, 1990].

2. 3. *C. perfringens* Type A Enterotoxin

Irrefutable evidence that *C. perfringens* was the aetiological agent of some food poisoning outbreaks came from the demonstration that all clinical manifestations of the disease could be induced with a crude extract of sporulating enterotoxin positive cells [*Stark & Duncan*, 1971]. The importance of the sporulation associated enterotoxin molecule was revealed when oral administration of the pure enterotoxin (CPE) produced typical food poisoning symptoms in previously healthy volunteers [*Skjelkvåle & Uemura*, 1977]. CPE toxicity has also been implicated in several cases of infectious diarrhoea. The symptoms were similar to those of *C. perfringens* food poisoning but more persistent, lasting up to seven days, and occurred in the absence of contaminated food [*Larson & Borriello*, 1988]. An opportunistic infection was suspected as the illness only affected hospitalised, mainly elderly, patients undergoing antibiotic treatment.

Initial studies of CPE produced misleading results, artifacts, due to the poor quality of the toxin preparations. Extensive biochemical and mode of action studies became feasible with the development of an improved purification method [*Granum & Whitaker*, 1980].

CPE is a single, highly hydrophobic (43% of the residues are hydrophobic), polypeptide comprising of 309 amino acids, molecular 35.4 kDa, and an isoelectric pH of 4.3 [*Granum & Skjelkvåle*, 1977]. The amino acid sequence has

been determined by peptide analysis [*Richardson & Granum*, 1985] and by the conceptual translation of the nucleotide sequence for a cloned CPE gene [*van Damme Jongsten*, 1989]. A single cysteine residue at position 186 provides the peptide with a free sulfhydryl group. The lack of stabilising disulfide bridges, due to the absence of cystine residues, is reflected in the heat lability of the peptide (rapidly inactivated at temperatures higher than 53°C) [*Granum & Skjelkvåle*, 1977].

Predictive models of CPE secondary structure from knowledge of the primary sequence were highly inconsistent but they did show concordance in two places. All models predicted the formation of α-helices by N-terminal residues 10 through to 25 and residues 195 to 215 [*Granum & Stewart*, In Press]. Biochemical evidence [*Richardson & Granum*, 1983; *Granum & Richardson*, 1991] supporting the existence of the first helix has been obtained. Trypsin digestion cleaves CPE in, at least (see below) two places, amino acids 15 and 25, both lysine residues which would be vulnerable to digestion if the peptide had adopted the α-helix conformation. The trypsinised CPE is deprived of two short, 10 and 25 residue, peptides which constituted its 25 N-terminal amino acids but they do appear to remain associated with the main 284 amino acid peptide [*Richardson & Granum*, 1983]. Support for the presence of the N-terminal helix *in vivo* came from the observation that digestion with the gut enzymes trypsin and chymotrypsin causes a 3-fold increase in CPE toxicity [*Richardson & Granum*, 1983; *Granum & Richardson*, 1991].

The guanidine hydrochloride and sodium dodecyl sulfate induced changes in the tertiary structure of CPE, examined using UV differential spectroscopy [*Granum & Whitaker*, 1980] and by measuring changes in the accessibility of the 17 ε-amino groups and the single α-amino groups to modifying chemicals [*Whitaker & Granum*, 1980], led to the formulation of a two domain model for CPE.

The physiological effects of CPE, investigated using perfused rabbit ileal loop assays [*McDonel*, 1974; *McDonel & Duncan*, 1975], are a net increased secretion of fluid, sodium and chloride ions with a concomitant reduced uptake of glucose. The cytological effects detected using Vero (African Green Monkey) cells,are selective alterations in membrane permeability, indicated by the leakage of ions and small molecules (Cr^{51}, nucleotides and lactate dehydrogenase) but not immediate loss of macromolecules such as RNA [*McClane & McDonel*, 1980; *Skjelkvåle et al.*, 1980; *McClane et al.*, 1988]. The protection against CPE cytotoxicity afforded by osmotic stabilizers such as sucrose, polyethylene glycol and bovine serum albumin, further advanced claims for the cell membrane as the primary site of CPE activity [*McClane*,

1984]. Other investigations of CPE mode of action including CPE binding studies found that CPE activity comprises a reversible, temperature independent binding step, an irreversible temperature dependent ion transport alteration step and a final calcium ion dependent step resulting in cell death [*Horiguchi et al.*, 1985 and 1986; *McClane*, 1984].

A model for CPE cytotoxicity was formulated in which trypsin activated CPE [*Granum et al.*, 1981] binds, possibly via the amino groups [*Whitaker & Granum*, 1980], to a 50 kDa protein receptor [*McClane*, 1984] in the brush border membranes of epithelial cells. A conformational change in CPE, from mainly β-sheet to an α-helix structure, mediates an alteration in the permeability of the cell membrane to ions and small molecules. The resultant change in the intracellular ionic concentration, in particular [Ca^{2+}] [*McClane et al.*, 1988] leads to morphological changes (bleb formation) and loss of macromolecules such as nucleic acids and proteins [*Granum*, 1985; *McClane et al.*, 1988] causing cell death.

Support for the two domain model for CPE has come from recent studies into the mode of action of CPE [*Hanna et al.*, 1989 and 1991]. Competitive binding studies have found that a short C-terminal fragment, encompassing residues $CPE_{290-319}$, acted as a competitive antagonist of CPE cytotoxicity for the Vero cell physiological receptors, an indication that the C-terminus contains the membrane binding site. These observations are an indication that the loss of biological activity that occurs with chemical modification of ε-amino groups is due to the inhibition of CPE binding resulting from alteration of lysine residue 301. It had previously been suggested that lysine residues 77 and 79 were involved in CPE binding as they are in a highly hydrophilic region of the protein and they reside on an exposed surface of CPE, which makes them readily available to the modifying agents [*Granum & Stewart*, In Press.]. Support for the involvement of the C-terminal lysine residues is provided by the 3-fold increase in biological activity that results from chymotrypsination [*Granum & Richarson*, 1991] as these residues would be removed by such a digestion [*Granum & Stewart*, In Press.].

There is an 8-24 hour delay after ingestion of food contaminated with *C. perfringens* vegetative cells before the onset of the symptoms of food poisoning. The delay is due to the time required for the cells to reach the ileum where they sporulate. Lysis of the sporangium liberates the toxin into the small intestine [*Duncan et al.*, 1972] where it can be activated by the digestive enzymes [*Granum et al.*, 1981].

It was thought that CPE was a structural part of the spore coat [*Frieben & Duncan*, 1973] and was therefore a sporulation-specific gene product [*Duncan*

et al., 1972]. A belief supported by the common kinetics of sporulation and CPE production [*Labbe & Duncan*, 1977]. Also, heat activation stimulated both sporulation and CPE production [*Uemura et al.*, 1973; *Tsai et al.*, 1974]. Food poisoning strains were believed to produce excess CPE due to altered regulation of gene expression [*Labbe & Duncan*, 1977]. The isolation of coatless spore mutants [*Lindsay et al.*, 1985] which did not produce CPE seemed to provide genetic evidence supporting the spore coat hypothesis. Evidence contradicting the sporulation specificity of CPE production was provided by the production of CPE by non-sporulating mutants [*Goldner et al.*, 1986], the sporulating abilities of Ent⁻ strains and the lack of total concordance between sporulation frequencies and levels of toxin production [*Uemura et al.*, 1973; *Skjelvåle et al.*, 1979; *Craven et al.*, 1981]. Although it was suggested that the inability to detect CPE in some strains was due to limitations of the enterotoxin detection methods [*Granum et al.*, 1984]. The detection of inclusion bodies containing CPE was an indication that the enterotoxin was not a spore coat protein [*Löffler & Labbe*, 1986]. Further proof was provided by the inability to isolate CPE from the spore coats of Ent⁻ strains [*Ryu & Labbe*, 1989]. Recent investigations into the distribution of the CPE gene, *cpe*, [This publication; *van Damme Jongsten et al.*, 1989 and 1990] have cast further doubt on the essential nature of CPE. DNA hybridization studies with *cpe* specific probes have failed to detect the gene in all strains isolated during food poisoning outbreaks and the majority of isolates (94%) from farm animals lacked a gene for CPE.

The unusual nature of *cpe* expression is more apparent when the wide variations in sporulation frequencies and enterotoxin producing abilities are considered alongside the difficulties in obtaining complete clones of *cpe* (Chapter 8). Observations made during the cloning and sequencing of *cpe* (This publication) are indicative of the involvement of an extrachromosomal genetic element in *cpe* expression and distribution. Although the structural gene for CPE does not appear to be located on a plasmid [Chapter 6; *Phillips Jones et al.*, 1989] a conjugative plasmid mediated mobilization of *cpe* (**Section 2. 1.**) can not be ruled out. Also, the recent detection of IS elements upstream of the exotoxin A gene in *Pseudomonas aeruginosa* [*Pritchard & Vasil*, 1990] sets an interesting precedent for the involvement of mobile genetic elements in toxin gene expression.

More extensive discussions of *cpe* cloning and extrachromosomal genetic elements are provided elsewhere in this thesis.

CHAPTER 3
THE PROJECT

3. 1. The Project Aims

A great deal of effort and money is being put into researching the clostridia, not because they are amongst the most pathogenic organisms known to man but because some members of the genus have tremendous biotechnological potential (Chapter 1). The increased scientific input has resulted in significant advances in the understanding of *Clostridium* genetics. It is likely that a major consequence of the work will be the generation of suitable mutant strains and procedures for the genetic manipulation of many *Clostridium* species. In the mean time any pieces of information concerning the genetics of the genus, such as codon biases and consensus sequences for control elements, are valuable additions to the growing pool of knowledge.

This project aimed to investigate the genetics of *C. perfringens*, an important human pathogen (Chapter 2). The study intended to focus on one aspect of *C. perfringens* genetics, in particular the gene that encoded an enterotoxin, a 35.4 kDa protein, that had been identified as the aetiological agent of a mild form of food poisoning caused by toxin type A strains [*Duncan & Strong*, 1969]. The protein had been purified and its amino acid sequence elucidated but questions concerning its function and location within the cell remained unsolved (Chapter 2).

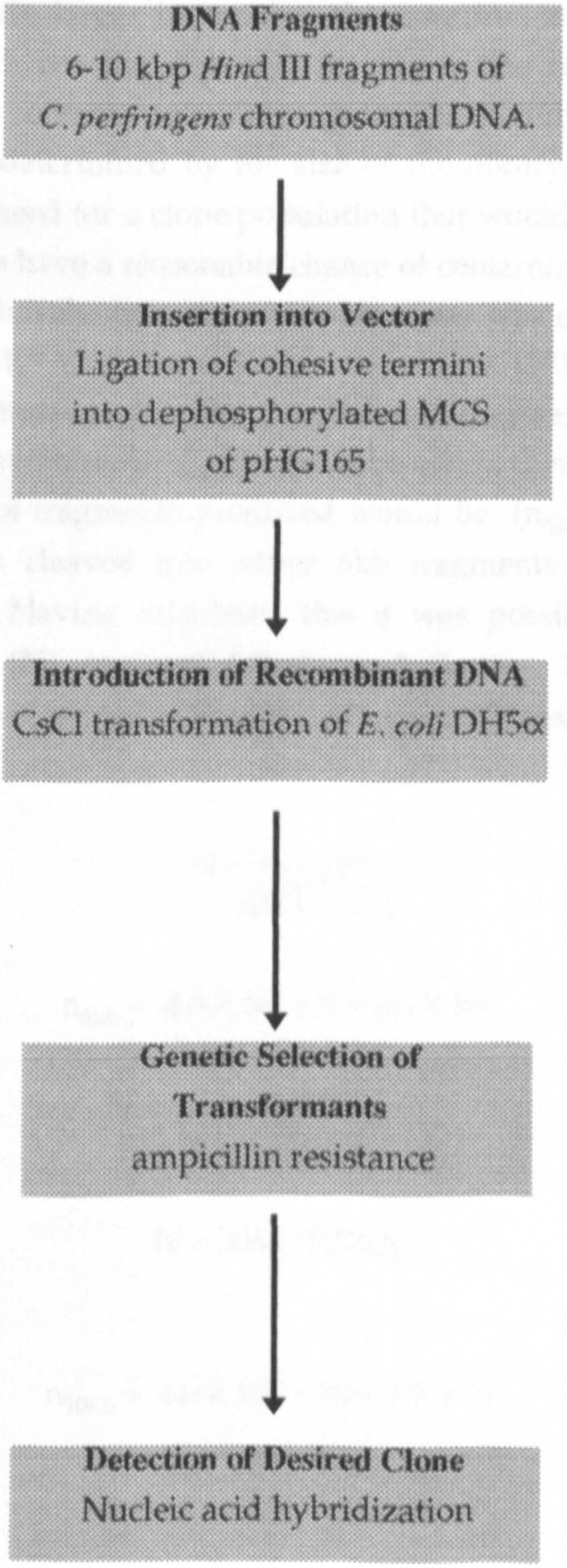
The initial purpose of the work was to produce an *Escherichia coli* clone containing the *C. perfringens* type A enterotoxin gene, *cpe*. A *cpe* clone could be utilised immediately in studies of enterotoxin function and gene expression. In the future it could be used in complementation and recombination experiments in *C. perfringens*.

Assuming faithful expression of *C. perfringens* genes in *E. coli* then the cloned *cpe* would provide large amounts of enterotoxin free of other contaminating *C. perfringens* proteins, including other toxins, which may confuse mode of action studies. Site-directed mutagenesis and deletion mutations of the cloned gene would provide specifically altered proteins useful for examining structure/function relationships. Hybridization experiments using a variety of *cpe* derived nucleic acid probes could examine a possible link between interstrain differences in expressivity of *cpe* (Chapter 2) with variation in genomic location and/or copy number.

Elucidation of the *cpe* DNA coding sequence would enable comparison between the theoretical translation product and the amino acid sequence

obtained by peptide analysis [*Richardson & Granum,* 1985]. Major discrepancies between the sequences may be an indication that the protein undergoes post-translational modifications. DNA sequence information would also reveal putative gene control elements which would be targets for future research into *cpe* expression.

3. 2. Proposed Cloning Strategy



3. 2. 1. Production of the DNA Fragments

A shotgun approach to the cloning of *cpe* was taken. In order to produce a genomic DNA library it was necessary to fragment the *C. perfringens* DNA into suitably sized portions. The actual fragment size required for library construction is dependent upon the cloning strategy. In this instance a 3.4 kbp pUC derived plasmid was the chosen cloning vector, which limited the maximum insert size to ≅ 10 kbp as transformation efficiency decreases dramatically with constructs over 15 kbp. Bacteriophage based vectors are able to accommodate larger inserts but these vectors are more difficult to contain and as such are not desirable for use in the construction of gene libraries liable to contain potentially pathogenic clones. The minimum fragment size was determined by the size of the library required. This was determined by the need for a clone population that would be manageable but also large enough to have a reasonable chance of containing the desired clone. If it was assumed that the genome of *C. perfringens* was equivalent in size to that of *E. coli* (4.0×10^3 kbp) *(now known to be 3.6×10^3 kbp [*Canard & Cole*, 1989]) and that the maximum randomly generated fragment size (F_{max}) would be 10 kbp whilst the minimum (F_{min}) would be 6 kbp; then the maximum and minimum number of fragments produced would be (n_{6kb}) and (n_{10kb}) if the entire genome was cleaved into either 6kb fragments (n_{6kb}) or 10 kbp fragments (n_{10kb}). Having calculated this it was possible to estimate the number of clones (N) required [*Clarke and Carbon*, 1976] to achieve a probability (P=99%) of obtaining the gene of interest (**Equation 3.1.**).

Equation 3. 1.

$$N = \frac{\ln (1-P)}{\ln (1- 1/n)}$$

For F_{min} :

$$n_{6kb} = 4.0 \times 10^3 \div 6 = 6.6 \times 10^2$$

$$N = \frac{\ln (1-0.99)}{\ln (1- 1/6.6 \times 10^2)}$$

$$N = 3065 \text{ *[2760]}$$

For F_{max} :

$$n_{10kb} = 4.0 \times 10^3 \div 10 = 4.0 \times 10^2$$

$$N = \frac{\ln (1-0.99)}{\ln (1- 1/4.0 \times 10^2)}$$

$$N = 1840 *[1656]$$

Where * is the adjusted figure based on the new size estimate for the *C. perfringens* genome.

Random fragments are generated either by mechanical shearing or restriction enzyme digestion of chromosomal DNA. The later method was employed for the cloning of the enterotoxin gene because the fragments produced from restriction enzyme digestion are easily inserted into complementary sites on the vector. The type II restriction endonuclease *Hin*d III was used because its AT rich recognition sequence (AAGCTT) was expected to appear frequently on the *C. perfringens* chromosome. Complete digestion would produce too small fragments but controlled partial digestion would produce overlapping DNA fragments in the desired 6-10 kbp size range. The use of overlapping fragments reduces the possibility of selectively excluding certain regions of the genome from the library and increases the chance of finding a clone with the required gene. This is because it should be on several restriction fragments in the starting pool.

3. 2. 2. Insertion into the Vector

The cloning vector pHG165 was cleaved at the single *Hin*d III site in the multiple cloning site (Appendix I) to produce a linearised DNA molecule with cohesive termini complementary to those of the similarly digested chromosomal DNA. Insertion of the chromosomal fragments into the vector occurred when the two species were mixed in the correct ratios because at suitable temperatures sticky ends reassociate and hydrogen bond formation results in the creation of 'nicked' DNA duplexes. The nicks were sealed by the action of DNA ligase (Appendix III). The ligation reaction temperature, 16°C, was a compromise between the enzyme optimum of 37°C and the dissociation temperature of the sticky ends [*Dugaicyzk et al.*, 1975; *Ferritti & Sgaramella*, 1981]. Intermolecular interactions were favoured over intramolecular ones by keeping the reaction volume to a minimum and preventing concatermerisation, which decreases the concentration of vector ends, by prior dephosphorylation of the linearised vector (Appendix III).

3. 2. 3. Introduction of the Recombinant DNA Molecules into the Host Cells

Exponentially growing cultures of the *E. coli* host cells, which are not naturally transformable with naked DNA, were washed with $CaCl_2$, a treatment known to make *E. coli* competent to take up exogenous DNA [*Mandel & Higa*, 1970; *Cohen et al.*, 1972]. The widely used heat shock transformation protocol [*Lederberg & Cohen*, 1974] (Appendix IV) was employed to introduce the recombinant plasmids into competent *E. coli* DH5α cells.

3. 2. 4. Genetic Selection of Transformants

3. 2. 4. 1. Ampicillin Resistance

The inherent inefficiency of the transformation process (maximum transformation efficiencies of 10^8 transformants per μg of supercoiled pBR322 DNA ≅ 1 transformant for every 1000 plasmid molecules) meant that it was advisable to employ a cloning vector such as pHG165 which carried a selectable marker like the the antibiotic resistance gene, *amp*^{r}. The incorporation of ampicillin into the agar actively discriminated in favour of transformed cells. *amp*^{r} encodes the enzyme β-lactamase which is secreted into the periplasmic space, the ampicillin site of action, where it catalyses the hydrolysis of the β-lactam ring [*Davis et al.*, 1980].

3. 2. 4. 2. α-Complementation

Screening of the transformants for those which possessed recombinant plasmids was facilitated by the use of the α-complementation system. The untransformed *E. coli* carried the *lacI* regulatory region but they were unable to produce a functional β-galactosidase because the *lacZ* lacked the codons for the N-terminal amino acids 11-41 thus only producing the carboxy-terminal, conferring a Lac$^-$ phenotype on the cells. Like most cells used in this assay system the DH5α strain carries the altered β-galactosidase gene, *lacZΔM15*, on an F' episome [*Ullman & Perrin*, 1970]. The transforming vector pHG165 also carried the regulatory *lacI* region and an altered β-galactosidase gene, *lacZ'*, which encoded a non-functional enzyme. The *lacZ'* produces a peptide consisting of 146 N-terminal amino acids of the enzyme which is able to complement, by association, the host encoded peptide to produce a functional β-galactosidase thereby restoring a wild type Lac$^+$ phenotype to the transformed cells.

The *lacZ'* and the *lacI* on pHG165 were interpolated by a short in-frame polylinker which did not interfere with expression of *lacZ'* . α-complementation was however abolished in those transformed cells which contained recombinant plasmids. The cells were Lac$^-$ because transcription of

lacZ' was prevented by the large insertions into the cloning site. The Ap^R transformants were visually screened for Lac^- colonies on MacConkey agar. The lactic acid produced by the fermentation of lactose turned the indicator in the medium red therefore the white Lac^- colonies were easily visible against the red Lac^+ background. The more frequently used media containing IPTG (isopropylthio-β-D-galactoside) the gratuitous inducer of the β-galactosidase gene in conjunction with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) a chromogenic substrate of the enzyme were not used since they may have induced expression of toxin genes contained on the inserted DNA. The $lacI^{qts}$ genotype of the uninduced cells ensured that any *lacI* controlled expression of insert DNA was minimal.

3. 2. 5. Detection of the *cpe* Clone

The enterotoxin gene would not predictably confer a selectable phenotype on the *E. coli* host cells so the Ap^R Lac^- colonies were screened for Cpe^+ transformants. Enterotoxin detection kits were commercially available and could have been incorporated into the screening regime but they were expensive, impractical and unreliable. Also it was unrealistic to assume that the required constitutive expression of the cloned gene in *E. coli* would occur, especially since *cpe* expression in *C. perfringens* cells appeared to be controlled. Plus, the considerable divergence between the codon biases in *E. coli* and *Clostridium* species (Chapter 1) meant that faithful translation into the protein could not be relied upon. Oligonucleotide probing of Ap^R Lac^- colonies was the only feasible means of primary screening large numbers of transformants. The theoretical DNA sequence for a short length of the published primary structure of the enterotoxin provided the basis for the oligonucleotide probe (Chapter 4). The redundancy of the mixed oligonucleotide population was minimised by choosing a region rich in amino acids which had only one or two codons and also by assuming that G-T interactions are stearically neutral. In this way a probe, which should be long enough to detect a unique sequence but not so long that it reaches an unacceptable level of redundancy, was synthesised several bases longer than the usual optimum of 17 bases.

3. 3. Proposed Sequencing Strategy

It was proposed that the chain termination method [*Sanger et. al.*, 1977], a method already well established in the laboratory, would be used in the DNA sequence determination of the enterotoxin gene. Premature termination of DNA synthesis, the major problem associated with this method of sequencing, had been overcome to a large extent by the complementing use of

the Klenow fragment of DNA polymerase I with avian reverse transcriptase [*Mierendorf & Pfeffer*, 1987] (Appendix III). Advice from a resident expert in the Maxam-Gilbert DNA sequencing method was available should the Sanger method have proved unworkable.

It was not envisaged that suitable computing facilities would become available before the sequencing programme was initiated so it was not likely that a random approach to the DNA sequence determination of *cpe* would be taken. The choice of strategy was therefore between the two direct approaches.

3. 3. 1. The Maxam-Gilbert DNA Sequencing Method [*Maxam & Gilbert*, 1977]

Firstly both strands of the target DNA are 5' end labelled. They are dissociated and separated into light and heavy strands by gel electrophoresis. The strands are then aliquoted out and subjected to different chemical modifications which cause the DNA strands to preferentially break at particular residues under certain conditions. The DNA sequence is determined by the relative positions of the broken radiolabelled strands separated by polyacrylamide gel electrophoresis and visualised by autoradiography.

3. 3. 2. The Chain Termination Method

The chain termination method capitalizes on two properties of DNA polymerases. Firstly their ability to faithfully synthesis complementary copies of single stranded DNA templates and secondly to use 2'3'-dideoxynucleoside triphosphates as substrates. Incorporation of the base analogues into the growing strand prevents continued DNA synthesis because they lack a 3' hydroxyl, the substrate for further chain elongation.

Four separate DNA sequencing reactions, one for each base, produce populations of mixed length DNA molecules with common 5' ends and base specific 3' ends. Each reaction mix contains all of the four bases, one or more of which are radiolabelled, and only a low concentration of one of the dideoxynucleosides, to enable some DNA synthesis before base specific chain termination occurs. The DNA polymerases, plus cofactors, cannot initiate DNA synthesis unless the ssDNA template is annealed to a complementary oligonucleotide primer. Primer design and specificity are crucial to prevent multiple priming of DNA synthesis which results in 'unreadable' autoradiographs. As for the Maxam-Gilbert method the DNA sequence is determined from the relative PAGE mobilities of the radiolabelled ssDNA strands.

3. 3. 3. Random and Direct Approaches to DNA Sequence Determination

A random approach to DNA sequencing requires subcloning of the target DNA. A pool of DNA fragment is created by digestion of the target DNA with several restriction enzymes. The fragments are then shotgun cloned into a suitable derivative M13 bacteriophage [*Messing et al.*, 1981]. M13 phages are filamentous, male-specific coliphages. They possess a single circular ssDNA chromosome so DNA sequencing of the inserts simply requires the isolation of recombinant chromosomes and initiation of DNA synthesis from an M13 universal primer. All the sequencing data is accumulated and analysed on a computer. The positions of the subcloned fragments are not pre-determined. Directed sequencing strategies systematically sequence the target DNA and the location or strand identity of each DNA fragment with respect to the starting DNA is known. Sequencing proceeds either via the progressive use of sequence deduced primers or by the sequencing of nested sets of deletion mutants from a universal primer. The later are generated by the subcloning of DNA fragments produced by the progressive and predictable digestion of one end of the target DNA. Suitable enzymes are BAL 31, pancreatic DNase I [*Hong*, 1987] or more usually exonuclease III [*Sambrook et al.*, 1989].

3. 4. DNA Hybridization

DNA hybridization was used on many occasions during the course of the studentship. Colony hybridization was used to identify clones containing recombinant plasmids and Southern blot analysis was used to investigate the distribution and architecture of the enterotoxin gene. The DNA probes were either chemically synthesised oligonucleotides or were restriction enzyme fragments derived from cloned *C. perfringens* DNA.

3. 4. 1. Colony Hybridization

Primary screening of transformants was performed by the *in situ* hybridization of oligonucleotide probes to lysed bacterial colonies [*Grunstein & Hogness*, 1975]. Experimental details, modified over time (Appendix V) basically consisted of the following. Colonies were overlaid with a filter and its orientation marked. The filters were lifted and placed onto a series of filter papers, soaked in various solution, which caused lysis of the adhered cells followed by denaturation and fixing to the filter of the released DNA. The filters were then washed well to remove cell debris, which could bind the probe, and prehybridized to block non-specific DNA binding sites on the filter. They were then placed in a solution containing a ^{32}P labelled probe and left to hybridize overnight. The filters were then washed to remove unbound

probe. Specifically bound probe was detected by autoradiography. Dark spots on the X-ray film were matched up to the corresponding colonies, which were then picked for further analysis.

3. 4. 2. Southern Blots

The distribution, genomic location and gene structure of *cpe* were examined using the Southern blot technique [*Southern*, 1975].

The DNAs of interest were subjected to agarose gel electrophoresis (Appendix III) followed by denaturation in sodium hydroxide solution, then blotted onto nitrocellulose or nylon membrane filters. The transfer of large DNA fragments was aided by depurination of the DNA, achieved by presoaking the gels in dilute hydrochloric acid. Initial blotting experiments required overnight blotting but the acquisition of blotting apparatus shortened the time needed for this procedure to just a few hours. Blotting was deemed to be successful if fluorescent bands corresponding to the ethidium bromide stained bands in the agarose gel could be seen on the filter when viewed under UV light. The filters were then washed to remove traces of agarose gel and the DNA fixed to the membrane. Prehybridization and hybridization was the same as for the colony blots.

3. 4. 3. Hybridization Conditions

Hybridization conditions were modified according to the filter material, the nature of the DNA probe and the experimental requirements i.e. level of stringency. All hybridizations were performed using relaxed criterion. Wash stringency levels were gradually increased after autoradiographic examination of the filters.

3. 4. 3. 1. Criterion for Permissive Hybridization and Stringency Washing

The rate of hybridization depends upon the nucleation frequency which in turn is affected by the concentration and nature of the probe, the ionic strength of the hybridization buffer and incubation temperature. Washing is dependent on the thermal stability (T_m) of the hybrid duplexes, which is affected by the ionic strength of the buffer, the base composition of the probe and the presence of denaturing agents. Only T_m measurements of DNA duplexes in solution have been obtained but observations of filter hybridizations indicate that the T_m for filter bound hybrids is much lower [*Kafatos et al.*, 1979].

For ssDNA probes major problems are only met at high concentrations (100ng ml^{-1}) when probe binds nonspecifically and irreversibly to the filter. Reassociation is only a problem when the probe possesses extensive regions of self-complementarity. Double-stranded DNA probes are able to hybridize

to complementary filter bound sequences (C_f) and sequences in the hybridization solution (C_s), therefore the relative concentrations of C_f and C_s are important factors in determining which of the two reaction will be favoured. Other important parameters are the probe complexity and incubation time. Prolonged incubation results in more and more reassociation of the probe and may result in leaching of bound probe from the filter. An estimate of the ideal incubation period (n) can be obtained from the relationship between the amount of probe added (X μg), the reaction volume (Y ml) and probe complexity (Z kbp) (probe length is a good enough approximation) [*Anderson & Young*, 1985] **(Equation 3.2.).**

Equation 3. 2.

$$n = \frac{1}{X} \times \frac{Y}{10} \times \frac{Z}{5} \times 2$$

The molecular weight of the probe also affects the rate of hybridization but the exact relationship is not understood. The base composition only affects the rate slightly with increase in rate concomitant with increasing %G + C. When %G + C is between 30% and 75% it has a large effect on the T_m. An estimate of the size of this effect has been made by combining data from several thermal stability studies of perfectly matched DNA-DNA hybrids in solution [*Anderson & Young*, 1985] (Equation 3.3).

Equation 3. 3.

$$T_m + 81.5 + 16.6 (\log M) + 0.41 (\%G + C) - 0.72 (\%\text{formamide})$$

Where M is the molarity of the monovalent cations up to a maximal of 1.0-2.0 M NaCl. The affect of formamide is to alter the stringency of the hybridization by decreasing the T_m. At certain concentrations it may also affect the rate of hybridization. The reduction in T_m of 0.72°C per 1% formamide is a compromise between the observed 0.5°C for poly(dG:dC) compared to 0.75°C for poly(dA:dT) [*Casey Davidson*, 1977]. Not surprisingly the rate of hybridization and hybrid stability are affected by the amount of mismatching both between probe and target DNA and probe to probe (for probes that contain imperfect repeated sequences). Hybridization incubation temperatures T_i take some account of the T_m and are always at least 20-25°C below it. In general, T_i. is 68°C (42°C with formamide) for well matched hybrids and 50-60°C (35-42°C) i.e. relaxed criterion for poorly matched

hybrids. The stability and rate of formation of mismatched duplexes increases with increasing ionic strength of the hybridization buffer. Filter bound hybrids formed under conditions of high ionic strength have to be subjected to low stringency washes. Wash buffers with low concentrations of salt cause dissociation of unstable hybrids.

3. 4. 3. 2. Additional Hybridization Buffer Reagents

Denhardt's reagent and milk powder [*Sambrook et al.,* 1989] are blocking agents, that is they prevent non-specific binding of the probe to the filter by blocking unfilled nucleic acid binding sites. Dextran sulphate is an inert polymer which increases the rate of hybridization by reducing the effective volume (Y ml **Equation 3.3.**) and whence increasing the probe concentration. The precise effect has not been determined therefore it should only be used it qualitative hybridizations because of difficulties in quantifying the results.

3. 5. Project Achievements

A genomic library was constructed in *E. coli* DH5α (Lac$^-$, Rec$^-$) by shotgun cloning a partial *Hin*d III digest of chromosomal DNA from *C. perfringens* strain NCTC 8239, a strain apparently devoid of extrachromosomal elements. The cloning vector pHG165 [*Stewart et al.,* 1986], a pUC derived plasmid but under *rop* copy number control, carried an ampicillin resistance selectable marker and, to enable α-complementation of the hosts mutated β-galactosidase, a *lacI* controlled *lacZ'*, interpolated with a polylinker. The Ap^R transformants were selected on ampicillin/MacConkey agar, to avoid induction of *C. perfringens* genes, and visually screened for transformants containing recombinant plasmids i.e. white, Lac$^-$ colonies. A single clone was detected using a degenerate oligonucleotide primer derived from the amino acid sequence of the enterotoxin. The plasmid DNA isolated from the clone appeared to contain several, possibly related plasmids, with a copy number different to that of the cloning vector. One of the plasmids, pLW1, was found to contain 90% of the DNA coding region (289 codons from a possible 321) for the *C. perfringens* type A enterotoxin, *cpe*, plus 6 kbp of 5' flanking DNA, inserted into the multiple cloning site of pHG165. None of the other plasmids hybridized the probe and no further analyses of them were performed.

A direct approach to the determination of the *cpe* DNA sequence was adopted. The sequence was elucidated using the dideoxy chain termination method with progressive, sequence deduced primers. The location of *cpe* was not known at the beginning of the sequencing project, so sequencing was initiated from a primer devised in a similar manner to the *cpe* oligonucleotide

probe. Conceptual translation of the *cpe* coding region was found to correspond well to the published amino acid sequence [*Richardson & Granum*, 1985], differences being mainly in those problem areas identified by Richardson and Granum. An appropriately positioned ribosome binding site i.e. a Shine-Dalgarno consensus sequence was located 5' to the ATG translation initiation codon. The upstream region also contained sequences with significant homologies to the putative transcriptional control elements, the Pribnow box and -35 sequence, of the tetanus toxin gene of *C. tetani* [*Eisel et al.*, 1986] and the type II β-lactamase gene of *Bacillus cereus* [*Sloma & Gross*, 1983].

Two direct repeats, separated by about 750 bp, of a sequence with homology to the cross-over point in site I of the Tn*3 res* region were found. One was located within the coding region whilst the other was 5' to the putative -35 sequence.

Restriction enzyme fragments of the cloned enterotoxin gene were used as probes to study interstrain variation in *cpe* architecture. Probe hybridization to Southern blotted chromosomal DNA was not observed for many of the *C. perfringens* type A strains or any of the non-type A strains. The probes did not detectably bind to plasmid DNA isolated from several food poisoning associated strains. There was no observable variation in enterotoxin gene architecture between those strains which did hybridize the probes.

Information from the hybridization studies concerning the architecture of *cpe* was utilised in the many unsuccessful attempts to clone the DNA coding for the enterotoxin C-terminus and the 3' flanking regions. These attempts employed different restriction fragments of *C. perfringens* DNA, alternative host cells and cloning vectors and altered transformation procedures. A second copy of the coding region for the N-terminus, this time on a shorter *Eco* RI/*Pst* I insert, was obtained but a clone containing the similarly sized contiguous *Pst* I/*Eco* RI fragment, which should have encoded the C-terminus, was not found.

An earlier attempt to clone the whole gene as an ≅ 10 kbp *Eco* RI fragment produced several thousand transformants containing recombinant plasmids. Analysis of some of the clones showed that they contained inserts in the correct size range. Colony hybridization identified several different clones which contained a copy of a *Hin*d III fragment located ≅ 2.5 kbp 5' to the ATG codon of the cloned portion of *cpe* but none that contained the gene. Several *Eco* RI fragments, equivalent to the insert size in the clones plus extra bands outside the cloning limits, hybridized the *Hin*d III fragment when it was used to probe Southern blot of chromosomal DNA from *C. perfringens* strain NCTC

8239. The altered hybridization patterns observed for the other strains were consistent with the fragment either being totally absent, most strains, or present in multiple copies around the genome.
The possible significances of these findings with respect to the observed inter and intrastrain variation in *cpe* expression are put forward in the Discussion section. Also presented are experiments which would test the hypotheses.

EXPERIMENTS

CHAPTER 4

CLONING THE ENTEROTOXIN GENE FROM *C. PERFRINGENS* TYPE A.

4. 1. Introduction

The enterotoxin gene cloning programme was instigated to provide a route for the elucidation of the biology of *C. perfringens* type A food poisoning (Chapter 3). It would also provide useful information concerning the genetics of a commercially valuable bacterial genus (Chapter 1).

A classical shotgun cloning strategy was adopted. Genetic containment requirements, due to the potential pathogenic nature of the resultant clones, placed constraints on the choice of host cell and cloning vector (Chapter 3). They also necessitated the use of a category 3 containment facility.

The *C. perfringens* genomic library was constructed and a potential enterotoxin gene clone identified by M. Routledge, the postgraduate student initially assigned to the project. The plasmid DNA was isolated from this clone and stored until the programme was recommenced.

The DNA solution was analysed by restriction enzyme digestion and appeared to be a mixed population of plasmid DNAs. The DNA was used to retransform *E. coli* cells in an attempt to ascertain whether this observation was real or an artifact i.e. *in vitro* degradation of the DNA by nucleases. Three plasmid types with apparently intact pHG165 backbones and a clone which had a restriction enzyme digest pattern similar to the original solution i.e. contained more than one plasmid species, were isolated negating the DNA degradation hypothesis.

Screening of the plasmids by Southern blot analysis with a degenerate oligonucleotide probe identified a presumptive *cpe* clone. A large scale plasmid extraction from the corresponding clone was prepared to enable further DNA analysis.

4. 2. Methods and Results

4. 2. 1. Bacterial Cultures

Chromosomal DNA was obtained from *C. perfringens* type A strain NCTC 8239 (Hobbs serotype 3), a strain known to produce high levels of enterotoxin [*Löffler & Labbe*, 1986]. The strain was maintained in CMM at room temperature (Appendix I). DNA was isolated from 500 ml, anaerobic, cultures in RCM (filtered to remove the agar) (Appendix I) grown overnight at 37°C.

E. coli host strains, JM107 and DH5α, were maintained as stab cultures (Appendix I). Competent JM107 cells were obtained from exponential stage cultures grown at 37°C in LB (Appendix I). Competent cells of DH5α were supplied ready competent and stored at -70°C until used.
Transformed *E. coli* cells (Appendix IV) were diluted in LB then spread onto MacAmp agar plates (Appendix I).

4. 2. 2. Production of *C. perfringens* Chromosomal DNA Fragments

Chromosomal DNA (strain NCTC 8239 had no detectable plasmids) was isolated from 1000 ml/500 ml of the overnight cultures using the previously described method [*Okita et al.*, 1981], modified to include an acetone pre-wash [*Heath et al.*, 1986] following difficulties in achieving lysis (Appendix II). The DNA was digested with various concentrations of *Hin*d III to determine the digestion conditions required to provide the optimum number of restriction fragments in the desired 6-10 kbp size range (Chapter 3). A large scale partial digest of the DNA was separated by preparative agarose gel electrophoresis (Appendix III) and gel containing the desired fragments excised. The DNA was recovered by electroelution and purified by passage through an ion exchange column (Appendix III).

4. 2. 3. Preparation of Recombinant Plasmids and Transformation Procedures

The *E. coli* cloning vector, pHG165 (Appendix I), was linearised by digestion with *Hin*d III and dephosphorylated with CAP (Chapter 3) (Appendix III). The *C. perfringens* DNA fragments were then ligated into the *Hin*d III site of the polylinker as previously described [*Stewart et al.*, 1986] (Appendix III). The unadulterated ligation reaction was used to transform *E. coli* JM107 using the CsCl transformation procedure (Appendix IV) or *E. coli* DH5α according to manufacturers instructions.

4. 2. 4. Primary Screening for *cpe* Clones

Cells were incubated for one hour at 37°C to allow expression of *Amp*^{r}. The Ap^R transformants were then selected on MacAmp agar plates incubated overnight at 37°C. They were then visually screened for white, Ap^R Lac^-, colonies to determine the size of the genomic library. *E. coli* JM107 cells transformed at a rate of 2×10^6 transformants μg^{-1} of supercoiled plasmid DNA but only produced a total of ≅ 500 recombinant clones (120/plate), too few to provide a reasonable probability of finding the desired clone (Chapter 3). The *E. coli* DH5α cells, which had a purported transformation efficiency of 10^8 μg^{-1} of supercoiled plasmid DNA, produced 4000 independent clones (1000/plate), a library sufficiently large to give a 99% of probability containing a *cpe* clone but not too large for primary screening by colony

hybridization.

A small number of clones were analysed to check that they contained recombinant plasmids with inserts in the desired size range. The library was then probed by colony hybridization with a [γ-^{32}P] end-labelled synthetic oligonucleotide. The probe sequence was derived from the conceptual DNA sequence for amino acids 9 through to 17 (**Figure 4.1.**) of the enterotoxin [*Richardson & Granum*, 1985]. This was the region with the least translational degeneracy but even this would have given a mixed oligonucleotide population with 512-fold degeneracy. This was reduced to only 4-fold by assuming that no stearic hindrance results from G-T mismatches i.e. G-T interactions were counted as neutral (M. Suissa personal communication to G. S. A. B. Stewart) (**Figure 4.2.**).

The colony hybridizations (Chapter 3) were performed at 42°C on nylon membranes [Gelman Sciences] according to the manufacturers instructions for permissive hybridization with formamide (Chapter 3). Low stringency washes of the filters, twice at 42°C for 30 minutes in 6 X SSC: 0.1% SDS and one 30 minute wash at 42°C in 2 X SSC: 0.1% SDS, were performed. The filters were then autoradiographed at -70% with double-sided X-ray film and an intensifying screen.

Only 1/4000 transformants hybridized the probe.

Figure 4. 1.

The Published Amino Acid Sequence Of The *C. perfringens* Enterotoxin With Its Predicted DNA Coding Strand Sequences.

								10						
Met	*Leu*	*Ser*	*Asn*	*Asn*	*Leu*	*Asn*	*Pro*	*Met*	*Val*	*Phe*	*Glu*	*Asn*	*Ala*	*Lys*
	T T	TCT	T	T	T T	T	T		T	T	A	T	T	A
ATG	CTC	AGC	AAC	AAC	CTC	AAC	CCC	ATG	GTC	TTC	GAG	AAC	GCC	AAG
	A	A			A		A		A				A	
	G	G			G		G		G				G	

20														30
Glu	*Val*	*Phe*	*Leu*	*Ile*	*Ser*	*Glu*	*Asp*	*Leu*	*Lys*	*Thr*	*Pro*	*Ile*	*Asn*	*Ile*
A	T	T	T T	T	TCT	A	T	T T	A	T	T	T	T	T
GAG	GTC	TTC	CTC	ATC	AGC	GAG	GAC	CTC	AAG	ACC	CCC	ATC	AAC	ATC
	A		A	A	A			A		A	A	A		A
	G		G		G			G		G	G			

								40						
Thr	*Asn*	*Asn*	*Asn*	*Leu*	*Ser*	*Asn*	*Ser*	*Asn*	*Leu*	*Ser*	*Asp*	*Gly*	*Leu*	*Tyr*
T	T	T	T	T T	TCT	T	TCT	T	T T	TCT	T	T	T T	T
ACC	AAC	AAC	AAC	CTC	AGC	AAC	AGC	AAC	CTC	AGC	GAC	GGC	CTC	TAC
A				A	A		A		A	A		A	A	
G				G	G		G		G	G		G	G	

	50												60
Val	*Ile*	*Asp*	*Lys*	*Gly*	*Asp*	*Gly*	*Leu*	*Ile*	*Leu*	*Gly*	*Glu*	*Pro*	*Ser*	*Val*
T	T	T	A	T	T	T	T T	T	T T	T	A	T	TCT	T
GTC	ATC	GAC	AAG	GGC	GAC	GGC	CTC	ATC	CTC	GGC	GAG	CCC	AGC	GTC
A	A			A		A	A	A	A	A		A	A	A
G				G		G	G		G	G		G	G	G

								70						
Leu	*Ser*	*Ser*	*Gln*	*Ile*	*Leu*	*Asn*	*Pro*	*Asn*	*Glu*	*Thr*	*Gly*	*Thr*	*Phe*	*Ser*
T T	TCT	TCT	A	T	T T	T	T	T	A	T	T	T	T	TCT
CTC	AGC	AGC	CAG	ATC	CTC	AAC	CCC	AAC	GAG	ACC	GGC	ACC	TTC	AGC
A	A	A		A	A		A			A	A	A		A
G	G	G			G		G			G	G	G		G

			80									90
Gln	*Ser*	*Leu*	*Thr*	*Lys*	*Ser*	*Lys*	*Glu*	*Val*	*Ser*	*Ile*	*Asn*	(*Val*	*Val*	*Gly*
A	TCT	T T	T	A	TCT	A	A	T	TCT	T	T	T	T	T
CAG	AGC	CTC	ACC	AAG	AGC	AAG	GAG	GTC	AGC	ATC	AAC	GTC	GTC	GGC
	A	A	A		A			A	A	A		A	A	A
	G	G	G		G			G	G			G	G	G

									100					
Phe;	*Ile*	*Gln*	*Ala*	*Ser*	*Val*	*Glu*	*Tyr;*	*Phe*	*Thr*	*Ser*	*Glu*	*Phe)*	*Thr*	*Ile*
T	T	A	T	TCT	T	A	T	T	T	TCT	A	T	T	T
TTC	ATC	CAG	GCC	AGC	GTC	GAG	TAC	TTC	ACC	AGC	GAG	TTC	ACC	ATC
	A		A	A	A				A	A			A	A
			G	G	G				G	G			G	

			110									120
Glu	*Arg*	*Ser*	*Val*	*Ser*	*Thr*	*Thr*	*Ala*	*Gly*	*Pro*	*Asn*	*Glu*	*Tyr*	*Val*	*Tyr*
A	A T	TCT	T	TCT	T	T	T	T	T	T	A	T	T	T
GAG	CGC	AGC	GTC	AGC	ACC	ACC	GCC	GGC	CCC	AAC	GAG	TAC	GTC	TAC
	A	A	A	A	A	A	A	A	A				A	
	G	G	G	G	G	G	G	G	G				G	

									130					
Tyr	*Lys*	*Val*	*Tyr*	*Ala*	*Thr*	*Tyr*	*Arg*	*Lys*	*Tyr*	*Gln*	*Ala*	*Ile*	*Arg*	*Ile*
T	A	T	T	T	T	T	A T	A	T	A	T	T	A T	T
TAC	AAG	GTC	TAC	GCC	ACC	TAC	CGC	AAG	TAC	CAG	GCC	ATC	CGC	ATC
		A		A	A		A				A	A	A	A
		G		G	G		G				G		G	

				140									150
Ser	*His*	*Gly*	*Asn*	*Ile*	*Ser*	*Asp*	*Asp*	*Gly*	*Ser*	*Ile*	*Tyr*	*Lys*	*Leu*	*Thr*
TCT	T	T	T	T	TCT	T	T	T	TCT	T	T	A	T T	T
AGC	CAC	GGC	AAC	ATC	AGC	GAC	GAC	GGC	AGC	ATC	TAC	AAG	CTC	ACC
A		A		A	A			A	A	A			A	A
G		G			G			G	G				G	G

									160					
Gly	*Ile*	*Trp*	*Leu*	*Ser*	*Lys*	*Thr*	*Ser*	*Ala*	*Asp*	*Ser*	*Leu*	*Gly*	*Asn*	*Ile*
T	T		T T	TCT	A	T	TCT	T	T	TCT	T T	T	T	T
GGC	ATC	TGG	CTC	AGC	AAG	ACC	AGC	GCC	GAC	AGC	CTC	GGC	AAC	ATC
A	A		A	A		A	A	A		A	A	A		A
G			G	G		G	G	G		G	G	G		

				170									180
Asp	*Gln*	*Gly*	*Ser*	*Leu*	*Ile*	*Glu*	*Thr*	*Gly*	*Glu*	*Arg*	*Cys*	*Val*	*Leu*	*Thr*
T	A	T	TCT	T T	T	A	T	T	A	A T	T	T	T T	T
GAC	CAG	GGC	AGC	CTC	ATC	GAG	ACC	GGC	GAG	CGC	TGC	GTC	CTC	ACC
		A	A	A	A		A	A		A		A	A	A
		G	G	G			G	G		G		G	G	G

									190					
Val	*Pro*	*Ser*	*Thr*	*Asp*	*Ile*	*Glu*	*Lys*	*Glu*	*Ile*	*Leu*	*Asp*	*Leu*	*Ala*	*Ala*
T	T	TCT	T	T	T	A	A	A	T	T T	T	T T	T	T
GTC	CCC	AGC	ACC	GAC	ATC	GAG	AAG	GAG	ATC	CTC	GAC	CTC	GCC	GCC
A	A	A	A		A				A	A		A	A	A
G	G	G	G							G		G	G	G

				200									210
Ala	*Thr*	*Glu*	*Arg*	*Leu*	*Asn*	*Leu*	*Thr*	*Asp*	*Ala*	*Leu*	*Asn*	*Ser*	*Asn*	*Pro*
T	T	A	A T	T T	T	T T	T	T	T	T T	T	TCT	T	T
GCC	ACC	GAG	CGC	CTC	AAC	CTC	ACC	GAC	GCC	CTC	AAC	AGC	AAC	CCC
A	A		A	A		A	A		A	A		A		A
G	G		G	G		G	G		G	G		G		G

									220					
Ala	*Gly*	*Asn*	*Leu*	*Tyr*	*Asp*	*Tyr*	*Arg*	*Ser*	*Ser*	*Asn*	*Ser*	*Tyr*	*Pro*	*Trp*
T	T	T	T T	T	T	T	A T	TCT	TCT	T	TCT	T	T	
GCC	GGC	AAC	CTC	TAC	GAC	TAC	CGC	AGC	AGC	AAC	AGC	TAC	CCC	TGG
A	A		A				A	A	A		A		A	
G	G		G				G	G	G		G		G	

				230									240
Thr	*Gln*	*Lys*	*Leu*	*Asn*	*Leu*	*His*	*Leu*	*Thr*	*Ile*	*Thr*	*Ala*	*Thr*	*Gly*	*Gln*
T	A	A	T T	T	T T	T	T T	T	T	T	T	T	T	A
ACC	CAG	AAG	CTC	AAC	CTC	CAC	CTC	ACC	ATC	ACC	GCC	ACC	GGC	CAG
A			A		A		A	A	A	A	A	A	A	
G			G		G		G	G		G	G	G	G	

									250					
Lys	*Tyr*	*Arg*	*Ile*	*Leu*	*Ala*	*Ser*	*Lys*	*Ile*	*Val*	*Asp*	*Phe*	*Asn*	*Ile*	*Tyr*
A	T	A T	T	T T	T	TCT	A	T	T	T	T	T	T	T
AAG	TAC	CGC	ATC	CTC	GCC	AGC	AAG	ATC	GTC	GAC	TTC	AAC	ATC	TAC
		A	A	A	A	A		A	A				A	
		G		G	G	G			G					

				260									270
Ser	*Asn*	*Asn*	*Phe*	*Asn*	*Leu*	*Val*	*Lys*	*Leu*	*Glu*	*Gln*	*Ser*	*Leu*	*Gly*	*Asp*
TCT	T	T	T	T	T T	T	A	T T	A	A	TCT	T T	T	T
AGC	AAC	AAC	TTC	AAC	CTC	GTC	AAG	CTC	GAG	CAG	AGC	CTC	GGC	GAC
A					A	A		A			A	A	A	
G					G	G		G			G	G	G	

									280					
Gly	*Val*	*Lys*	*Asp*	*His*	*Tyr*	*Val*	*Asp*	*Leu*	*Ser*	*Leu*	*Asp*	*Ala*	*Gly*	*Gln*
T	T	A	T	T	T	T	T	T T	TCT	T T	T	T	T	A
GGC	GTC	AAG	GAC	CAC	TAC	GTC	GAC	CTC	AGC	CTC	GAC	GCC	GGC	CAG
A	A					A		A	A	A		A	A	
G	G					G		G	G	G		G	G	

				290									300
Tyr	*Val*	*Leu*	*Val*	*Met*	*Lys*	*Ala*	*Asn*	*Ser*	*Ser*	*Tyr*	*Ser*	*Gly*	*Asn*	*Tyr*
T	T	T T	T		A	T	T	TCT	TCT	T	TCT	T	T	T
TAC	GTC	CTC	GTC	ATG	AAG	GCC	AAC	AGC	AGC	TAC	AGC	GGC	AAC	TAC
	A	A	A			A		A	A		A	A		
	G	G	G			G		G	G		G	G		

								309
Pro	*Tyr*	*Ser*	*Ile*	*Leu*	*Phe*	*Gln*	*Lys*	*Phe*
T	T	TCT	T	T T	T	A	A	T
CCC	TAC	AGC	ATC	CTC	TTC	CAG	AAG	TTC
A		A	A	A				
G		G		G				

Figure 4. 2.
Derivation of the base sequence for oligonucleotide *cpe probe*

A degenerate oligonucleotide probe was made based on the known amino-acid sequence of the enterotoxin. The region which would give the least degeneracy i.e. the one with the fewest possible base combinations, corresponded to amino-acids 9 through to 17.

9 10 11 12 13 14 15 16 17

N---met---val---phe---glu---asn---ala---lys---glu---val---C

Possible Bases at Each Position in the mRNA

```
                A           A           A
                U   U   A   U   U   R   A   U
5'-------AUG GUG UUC GAG AAC GCG AAG GAG GUG -------3'
                C           C           C
```

A Complementary DNA Strand with 512-fold Sequence Degeneracy

```
                T           T           T
                A   T   T   A   A   T   A   A
5'-------CAC CTC CTT CGC GTT CTC GAA CAC CAT-------3'
                G           G           G
```

Assume:

A.....pairs with.....T

A.....does not pair with.....C

G.....pairs with.....C

G.....not hindered by.....T

Sequence of theOligonucleotide Probe with Degeneracy Reduced to 4-fold

```
                            T           T
5'-------AC TTC TTT GGC GTT TTC GAA GAC CAT-------3'
```

Comparison to the Elucidated DNA Sequence

```
5'-------AC TTC TTT AGC ATT TTC GAA CAC CAT-------3'
```

4. 2. 5. Secondary Screening for *cpe* Clones

A large scale plasmid DNA isolation from the single positive colony, 'tox', was prepared and stored at 4°C, in TE buffer (Appendix I). A *Hin*d III digest of the 'tox' DNA indicated a mixed plasmid population (**Lanes 3 & 4. Photograph 4.1.**). To check if this interpretation was correct the 'tox' DNA was used to re-transform *E. coli* JM107 cells. Mini-prep plasmid DNA (Appendix II) from 12 colonies, picked at random, was digested with *Hin*d III and four clone types identified. Three contained single plasmid species (**Lanes 5 & 9; 7 & 11; and 8 & 12. Photograph 4.1.**), with different but similar patterns of restriction enzyme digestion, but the fourth clone again appeared to contain a mixture of the other plasmid types (**Lanes 6 & 10. Photograph 4.1.**).

A Southern blot (Chapter 3) (Appendix V) of *Hin*d III digested DNA from the four types of clone, along with *Hin*d III digested bacteriophage λ and pHG165 control DNAs (**Photograph 4.1. Top**), was prepared on a nitrocellulose filter [Gelman Sciences]. The filter was then probed with the end-labelled 26 bp oligonucleotide probe according manufacturers instructions. Washing and autoradiography was identical to the colony blots.

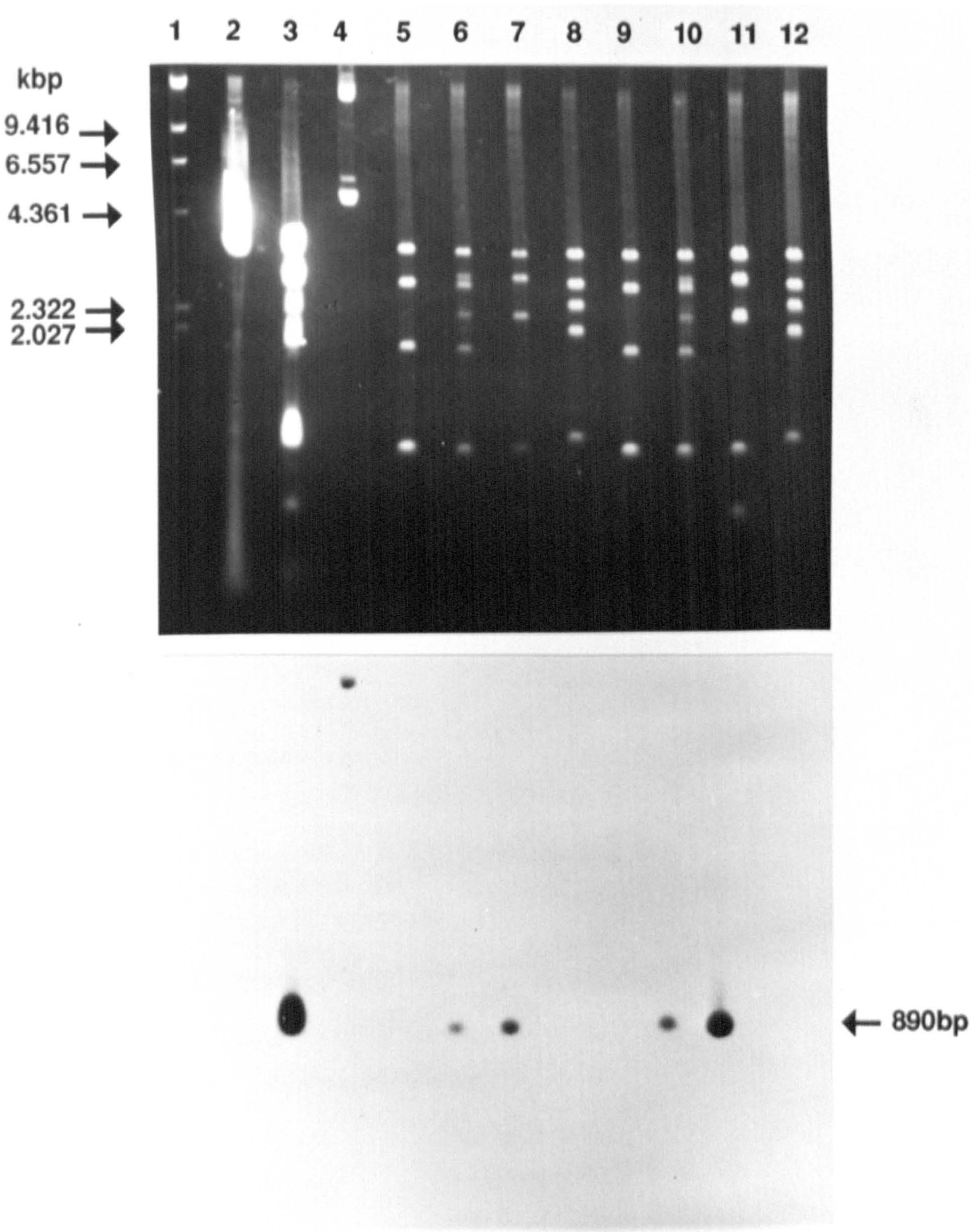
A single DNA band, corresponding to ≅ 900 bp, from the mixed plasmid population and an equivalent sized band from one of the other three clone types hybridized the probe (**Lanes 6 & 10 and 7 & 11. Photograph 4.1. Bottom**).

A large scale isolation of plasmid DNA, pLW1, from the clone with the single plasmid type was prepared and stored at 4°C.

Photograph 4. 1.
Southern Blot Identification of a Plasmid, pLW1, Presumed to be Carrying the Enterotoxin Gene

Lane	Description
1	*Hin*d III digested bacteriophage λ DNA. A size marker and negative control.
2	Uncut pHG165 also a negative control.
3	*Hin*d III digested 'tox' DNA. A positive control.
4	Uncut 'tox' DNA also a positive control.
5	*Hin*d III digested plasmid type 2.
6	*Hin*d III digested DNA from the multi-plasmid clone.
7	*Hin*d III digested pLW1.
8	*Hin*d III digested plasmid type 3.
9	As for lane 5.
10	As for lane 6.
11	As for lane 7.
12	As for lane 8.

The top photograph is the ethidium bromide stained 0. 8% agarose gel of the above DNAs, viewed under UV light. The gel was Southern blotted and hybridized with the oligonucleotide enterotoxin gene probe and autoradiographed, bottom photograph.

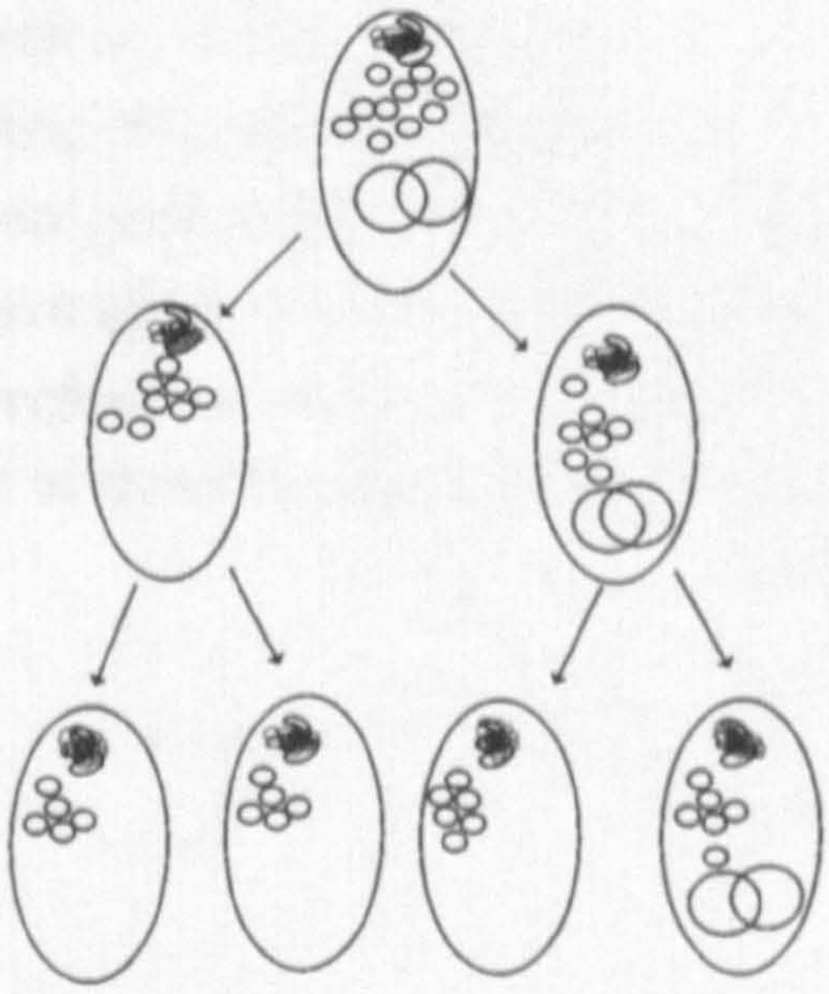

1 2 3 4 5 6 7 8 9 10 11 12
kbp
9.416
6.557
4.361
2.322
2.027
890bp

4. 3. Discussion

Two unexpected and inexplicable observations were made during the cloning of the enterotoxin gene.

Firstly, the *C. perfringens* DNA inserts were produced from a partial *Hin*d III digest, the recognition sequence for which should occur several times on a 6-10 kbp stretch of DNA, so the library should contain many overlapping fragments. An estimate, based on observations of the amino acid sequence for the enterotoxin [*Richardson & Granum,* 1985], placed the size of the *cpe* coding region at around 1 kbp, small enough to be contained on more than one of the fragments in the starting pool. So was it purely coincidence that only one *cpe* clone was identified from over 4000 transformants, 30 % more than the largest estimated number (140% more than the minimum) of clones needed to give a 99% probability of the library covering the whole the genome?

Secondly, the presence of more than one plasmid type in a single clone was most unusual. Transformation of *E. coli* is a rare event so it would be a very unusual occurrence for a single cell to be transformed by more than one plasmid. It would therefore be extremely improbable that three plasmids would independently enter the same bacterium. Even if it did happen they probably would not all be maintained unless they conferred different selective advantages on the host cell. Plasmids of different sizes replicate at different rates, large plasmids taking longer to replicate than smaller plasmids. When it is time for the cell to divide, the numbers of each plasmid type vary if, as in this case, they are not subject to copy number control. Asymmetrical segregation of the plasmids to the daughter cells results in some cells not having representatives of each plasmid type. This rapidly leads to a decrease in the proportion of cells containing large plasmids compared to those that have smaller plasmids, eventually resulting in loss of the larger plasmids from the population.



In this experiment the initial DNA preparation containing the mixed plasmid population could have arisen from the selection of more than one clone from the plate which had over 1000 white colonies. The subsequent independent isolation however of three clone types which carried only one plasmid species and a single clone type which appeared to contain the original three plasmids, but no intermediate clone types, from a plate which had relatively few colonies (≅ 300), would be evidence in favour of the effect being real as opposed to experimental error. The only other way, that could be thought of, to explain the observations was if the three plasmids were related e.g. DNA rearrangement of one of the three gave rise to two daughter plasmids. However *E. coli* DH5α are Rec⁻ so if DNA rearrangements did occur they must have been independent of the host recombination system e.g. a transposon mediated rearrangement. A transposition based explanation would also conform to the observation that the majority of the clones examined (11/12) had only one plasmid since unregulated transposition is deleterious to the host and is therefore usually repressed *Yin & Reznikoff*, 1988; *Yin et al.*, 1988].

Furthermore, anecdotal evidence for the presence of a *rop* independent origin of replication, needed for replicative transposition [Shapiro, 1979], came from the observation that the amount of plasmid DNA recovered from large and small scale extractions (Appendix II) was in accord with that expected for high copy number plasmids. All the evidence for and against the involvement of a mobile genetic element in enterotoxin gene expression is discussed in Chapter 10.

No attempts were made to ascertain whether or not the plasmids were related because only one of them, pLW1, appeared to carry *cpe*, the object of the investigation. It was later found (Chapter 5) that the *cpe* on pLW1 backed up to the cloning vector, pHG165. pHG165 appeared to be recovered intact from all three plasmids therefore, if they were related, DNA sequencing from the pHG165 multiple cloning site would possibly identify part of the 3' coding region of the enterotoxin gene in the other plasmids. Also it should be noted that even if DNA rearrangements did occur it does not necessarily reflect what happens in *C. perfringens* since the reactions of supercoiled plasmid DNA are very different to those of chromosomal DNA.

CHAPTER 5

SEQUENCING THE ENTEROTOXIN GENE

5. 1. Introduction

5. 1. 1. Reasons for Sequencing DNA

There are many reasons why knowledge of DNA sequences, not only coding regions but also untranslated sequences e.g. eucaryotic introns, can be useful. Most obvious is that it is a prerequisite for future DNA manipulation based projects. Computers enable rapid searching of DNA sequences to identify all known restriction enzyme sites, which aids the subcloning of defined regions such as the promoters or terminators and/or sequences which code for specific protein domains. More subtle advantages are available to investigators examining the effects of mutations e.g. how alteration of possible regulatory sequences affects levels of gene expression. Such projects progress more rapidly if candidate sequences, such as tandem or inverted repeats (common characteristics of control elements in the appropriate context) are identified from the start.

Conceptual translation of a DNA sequence into protein in all possible open reading frames (ORFs) (there are six ORFs for each dsDNA molecule, three for each strand) may clear up amino acid sequence ambiguities that have resulted from difficulties in protein purification or peptide sequencing. Also questions regarding post-translational modifications such as the removal of signal peptides may be answered.

If there is easy access to a 'user friendly' computerised data base then the DNA sequence and the deduced amino acid sequence can be compared with other sequences. Any similarities to DNAs and/or proteins of known function can reveal very useful information regarding possible functions or modes of action of the DNA under investigation e.g. otherwise unremarkable DNA sequences may be homologous to gene control regions from entirely unrelated organisms. The lack of any detectable homologies may be equally revealing. The matriculations used to perform the comparisons must be known and understood, otherwise the results from such surveys can be the subject of gross misinterpretation.

Finally taxonomic issues may be clarified and evolutionary inferences made on the basis of codon usage as well as the more obvious DNA and/or protein sequence homologies. DNA sequence homologies are assumed to be the result of common ancestry. Protein homologies that occur without corresponding homology at the DNA level may be the result of common ancestry or

convergent evolution, in either instance it is assumed there has been selection for a particular structure/function relationship.

5. 1. 2. Sequencing Strategies and Methods

The correct choice of sequencing strategy, either random or directed, is crucial to the success of any sequencing programme and is usually determined by the available expertise and facilities. Materials derived during the sequencing, primers and subclones, are often useful for future projects, and should also be taken account of when deciding upon a suitable strategy.

If a random approach (Chapter 3) is desirable then extensive computing facilities must be acquired before it becomes a feasible proposition. The advantages are that the accuracy of the sequence obtained is extremely high due to the number of times each part of the fragment is sequenced before the entire region of interest has been elucidated. In addition to this, the entire project can be completed using a universal primer, so there is no time gap between clone production and sequencing. The major disadvantage is that some sequences may be underrepresented and therefore difficult to locate within the library, adding time and complexity to the project.

Direct approaches (Chapter 3) require either the generation of one, or more reliably two, sets of nested deletions or the synthesis of sequence derived primers. In both cases the order of each sequence is indisputable so computer analysis is not necessary. The multiple primer route does require either access to an oligonucleotide synthesizer or sufficient funds for the purchase and rapid delivery of primers from external agencies.

There are two DNA sequencing methods (Chapter 3), both of which involve the generation and length determined separation by polyacrylamide gel electrophoresis of radiolabelled DNAs. The Maxam-Gilbert method [*Maxam & Gilbert*, 1977] which relies on the chemical degradation of asymmetrically labelled DNA (the labelling mechanism varying with the nature of the target DNA) has largely been replaced by the DNA polymerase based, dideoxy sequencing method [*Sanger et al.*, 1977]. This method involves the termination of enzyme driven DNA synthesis by the incorporation of dideoxynucleotides into the growing DNA strand, whence the terminology. Improved enzymes and refinements to the reaction conditions have resulted in the resolution of even the most difficult of sequencing problems via the dideoxy route.

The Klenow fragment of *E. coli* DNA polymerase I [*Sanger et al.*, 1977] and murine or avian reverse transcriptases [*Mierendorf & Pfeffer*, 1987] were the enzymes originally used for dideoxy sequencing. The Klenow fragment was used for the largest part of any sequencing project because although the

reverse transcriptases were very good sequencing enzymes their prohibitive costs meant that they were reserved for the resolution of problem sequences. Most of the difficulties encountered with Klenow were due to its sensitivity to contaminants, its low processivity and its difficulty in coping with regions of high secondary structure. A modified and a genetically engineered version of bacteriophage T7 DNA polymerase, Sequenase™ and Sequenase version 2.0, (the wild type enzyme has 3' → 5' exonuclease which is an undesirable characteristic for a sequencing enzyme) [*Tabor & Richardson*, 1987] and the DNA polymerase from the thermophilic bacterium *Thermus aquaticus*, *Taq* DNA polymerase, have recently replaced Klenow and the reverse transcriptases as the most commonly used DNA sequencing enzymes. The high processivity of the Sequenase enzyme means that non of the premature termination problems associated with Klenow are encountered, and the cloned version of the enzyme is easy to produce so there are no difficulties in obtaining large amounts at reasonable prices. The highly thermostable *Taq* DNA polymerase is useful for sequencing DNA that forms stable secondary structures at the normal T7 polymerase working temperature of 37°C [*Innis et al.*, 1988].

5. 1. 3. Sequencing *cpe*

Many of the reasons for sequencing DNA put forward in the first part of this chapter were pertinent to the argument for sequencing the enterotoxin gene. The confusion surrounding *cpe* expression and the function/mode of action of the protein meant that investigations not only of putative gene control elements but also the CPE coding region would feature prominently in future projects. The base sequence of the enterotoxin coding sequence and the flanking DNA would be necessary for these projects which would be centred around the subcloning and mutagenesis of *cpe*. Additionally, although Richardson and Granum [*Richardson & Granum*, 1985] had published an amino acid sequence for the enterotoxin molecule, they had also identified a sixteen amino acid region which had proven refractory to peptide sequencing. DNA sequencing would confirm the correctness of the amino acid sequencing data and clarify the order of amino acids in the aforementioned region. Finally, as discussed in Chapter 1, the clostridia are a large pleomorphic group of bacteria with very diverse ancestry. The genus could be subdivided on the basis of assumed evolutionary relationships when sufficient sequencing data becomes available to allow comparisons of codon usage etc. .

A direct sequencing strategy had to be adopted because of the lack of easy access to suitable computing facilities when the sequencing of *cpe* was

initiated. The acquisition of an oligonucleotide synthesizer weighted the choice of approach in favour of one which employed sequence deduced primers as opposed to the production and sequencing of nested deletions.
Most sequencing projects that adopt a progressive primer approach use a primer complementary to the cloning vector to initiate the sequencing. In order for this strategy to work DNA synthesis from the primer must run into the DNA of interest. This is accomplished by mapping the insert, thereby locating the gene with its flanking DNA, then subcloning it into a suitable vector. In this instance the need for subcloning and the prerequisite mapping of the insert was obviated by the synthesis and successful use of a degenerate primer, derived in the same manner as the oligonucleotide probe used to detect the clone (Chapter 4). Both strands of the available DNA of the enterotoxin gene were subsequently sequenced and analysed.

5. 2. Materials and Methods

5. 2. 1. Strategy

As discussed in the previous section, a wish to avoid the immediate need to compile a restriction enzyme map of pLW1 and subsequent subcloning of *cpe* meant that the usual universal primer would not be a feasible first primer. Therefore a novel primer (Primer A) based on the only available information, the amino acid sequence of the enterotoxin, had to be devised (**Figure 5.1a.**). Sufficient sequence information (**Figure 5.1b.**) was obtained using Primer A to enable a second primer (Primer B) to be composed without any mismatches (**Figure 5.1c.**). The sequencing from this primer indicated that the target DNA did code for the enterotoxin molecule and that the pursuit of a progressive, sequence deduced primer, sequencing strategy was a viable proposition. The oligonucleotide primers used to sequence *cpe* were numbered according to their order of synthesis and use (**Figure 5.3.**) The primer locations with respect to *cpe* and the amount of sequencing obtained from each is illustrated (**Figure 5.4.**).

5. 2. 2. Synthesis of the Sequencing Primers

The oligonucleotide primers were synthesised 3' → 5', using phosphoramidite chemistry on an automated DNA synthesizer [Cruachem or Milligen Biosearch] according to manufacturers instructions (Appendix I). The oligonucleotides were separated from their glass bead supports by overnight incubation in 1-2 ml of ammonia solution (specific gravity 0.88) at 55°C. They were then lyophilised and dissolved in TE buffer (Appendix I). The amount of primer was estimated by obtaining the optical density at 260 nM of 10 µl of

oligonucleotide solution in 1 ml of H_2O (in a 1 cm path glass cuvette). The primer concentration was calculated from the relationship that 1 $O.D._{260} \cong 33$ $\mu g\ \mu l^{-1}$ of ssDNA. A small amount ($\cong$ 1 µg) of primer in formamide [Sigma] and 10 mM EDTA (Appendix I) was assayed on a 16% polyacrylamide electrophoresis gel (Appendix III) to check the quality of the oligonucleotide preparations.

5. 2. 3. The Sequencing Template

A large scale plasmid preparation of pLW1 was extracted from *E. coli* DH5α cells and purified by equilibrium centrifugation in an CsCl-EtBr gradient [*Guerry et al.*, 1973] (Appendix II). The purified DNA was redissolved in TE buffer to a final concentration of 1 $\mu g\ \mu l^{-1}$.

5. 2. 4. The DNA Dependent DNA Polymerases

Most sequencing of the upstream region and the coding sequence for the N-terminal portion of the protein was performed using the Klenow fragment of *E. coli* DNA polymerase I [Amersham]. Sequences capable of forming secondary structures, such as cruciforms, often caused premature cessation of DNA synthesis by Klenow. The already low processivity of the enzyme was probably decreased further resulting in dissociation of the Klenow and the template. These regions of dyad symmetry along with other problem sequences were resolved using AMV (avian myelocytomatosis virus) reverse transcriptase [Boehringer].The prohibitive cost of this enzyme prevented its routine incorporation into the *cpe* sequencing programme. Sequenase, the bacteriophage T7 DNA dependent DNA polymerase, was introduced in the later stages of the sequencing project in the form of a DNA sequencing kit [Pharmacia]. The higher processivity of this enzyme, compared to the Klenow, coupled with the optimised reaction conditions provided by the kit meant that progress was rapid. Fewer blocks in the sequencing were encountered due to premature termination and the sequences obtained were easier to process due to a reduction, though not a total absence, of artifactual banding i.e. bands that occupy more than one of the sequencing tracks.

5. 2. 5. Sequencing Reactions

Whatever DNA polymerase was used, the radiolabelled nucleotide was always [α-^{35}S] dATPαS [Amersham].

The Klenow sequencing reactions (Appendix III) were as previously described [*Williams et al.*, 1986] whilst the AMV reverse transcriptase method and dNTP mixes were as described by Bartlett *et al.* [1986] (Appendix III). The

preparation of the DNA template/primer was the same for both Klenow and AMV reverse transcriptase catalysed reactions. The alkali denaturation of the double stranded plasmid DNA template was followed by addition of the oligonucleotide primer and neutralisation with sodium acetate. The annealing reaction was then dialysed against TE buffer [*Marusyk & Sergeant* 1980].

The T7 reactions were essentially as described in the manual for the T7 Sequencing™ Kit [Pharmacia]. Misincorporation of nucleotides by T7 DNA polymerase was further reduced by modifying the protocol to include a chase step. The chase cocktail contained terminal deoxynucleotidyl transferase [*Fawcett & Bartlett*, 1990] (Appendix III).

5. 2. 6. Sequencing Gels

The sequencing reactions were electrophoresed on 4 mm thick, denaturing, 8% polyacrylamide gels at 1200 V, 25 mA, for two, four or six hours (Appendix III). The gels were fixed in a solution of glacial acetic acid:methanol:water (1:1:8) for 30 minutes then dried either at 55°C for two hours or room temperature overnight. The gels were then autoradiographed, using single sided high performance autoradiography film [Hyperfilm™ - βmax. Amersham], for a minimum of twelve hours (exposure time varied with the radioactivity of the ^{35}S preparation). The films were developed according to manufacturers instructions and the DNA sequence determined from the relative positions of the bands in each of the four tracts (G, A, T, C). The same order of loading was adhered to throughout the programme.

Figure 5. 1.
Starting to Sequence *cpe*

5. 1a. A degenerate sequencing primer was made based on the known amino-acid sequence of the enterotoxin. The region would which would give the least degenerate primer i.e. the one with the fewest possible base combinations, corresponded to amino-acids 9 through to 14.

Amino-acids: 9 10 11 12 13 14

N-------met---val---phe---glu---asn---ala-------C

Possible mRNAs:

```
                        A               A
                        U   U   A   U   U
5'-------AUG GUG UUC GAG AAC GCG-------3'
                        C               C
```

i.e. equivalent to the coding DNA strand.

Assume:

A .....pairs with..... T

A .....does not pair with..... C

G .....pairs with.....C

G .....not hindered by.....T

Complementary DNA Strand and First Degenerate Primer (A):

```
5'-------GC GTT TTC GAA GAC CAT-------3'
```

i.e. equivalent to the non-coding DNA strand, therefore hybridizes to the coding strand initiating DNA synthesis towards the ATG methionine codon. Only a short sequence was obtained using this primer but it was sufficient to enable a second primer to be designed.

5. 1b. Sequence Obtained From First Primer and Corresponding Amino-Acids:

5' TT ACT AAG CAT ATTATA 3'
asn ser leu met upstream
4 3 2 1

5. 1c. Sequence of New Primer (B) to Read Back Into the Coding Region:

5' TATAAT ATG CTT AGT AA 3'

Figure 5. 3.
Primers Used In The Sequencing Of *cpe*

Sequence (5' → 3')	Corresponding *cpe* Bases
First possibly degenerate primer: A	
GC GTT TTC GAR GAC CAT	sequencing towards the ATG codon from base +41 → +25
Second possibly degenerate primer: B	
TATAA ATG CTT AGT AA	-6 → +11
Forward Primers:	
6. CAC TCT CCT TGT TTA TAA T	-310 → -293
5. GAT ATG TAT CCA AAA TA	-78 → -62
1. AAT TTA AAT CCA ATG GTG TTC	+13 → +33
3. ACA CCA ATT AAT ATT ACA AAC	+76 → +96
8. TTT ATA CAA GCA TCT GTA G	+283 → +301
9. GGT AAT ATC TCT GAT GAT	+438 → +455
10. CCA GCT GGT AAT TTA TAT GAT	+655 → +675
11. T AAT AAT CTA GTG AAA TTA GAA	+804 → +825
Reverse strand primers:	
lac α reverse primer	M13 sequencing primer
GTT TTC CCA GTC ACG AC	
14. TT AAA ATT ATT TGA ATA ATA AAT	+806 → +787
12. GTA TGA GTT AGA AGA ACG C	+696 → +678
13. TCC TAA GCT ATC TGC AGA T	+516 → +498
7. AAT TTG ACT TGA AAC TAC	+186 → +169
2. GTT TGT AAT ATT AAT TGG TGT	+96 → +76
4. GAT ATG TAT CCA AAA TA	-61 → -78
15. CAC TAA ACA TAA TAT TAT ATA	-272 → -293

Figure 5.3. and 5.4.

The sequencing primers were numbered according to order of synthesis and use. Primers together with the lengths of DNA sequence obtained from them are represented diagrammatically in Figure 5.4..

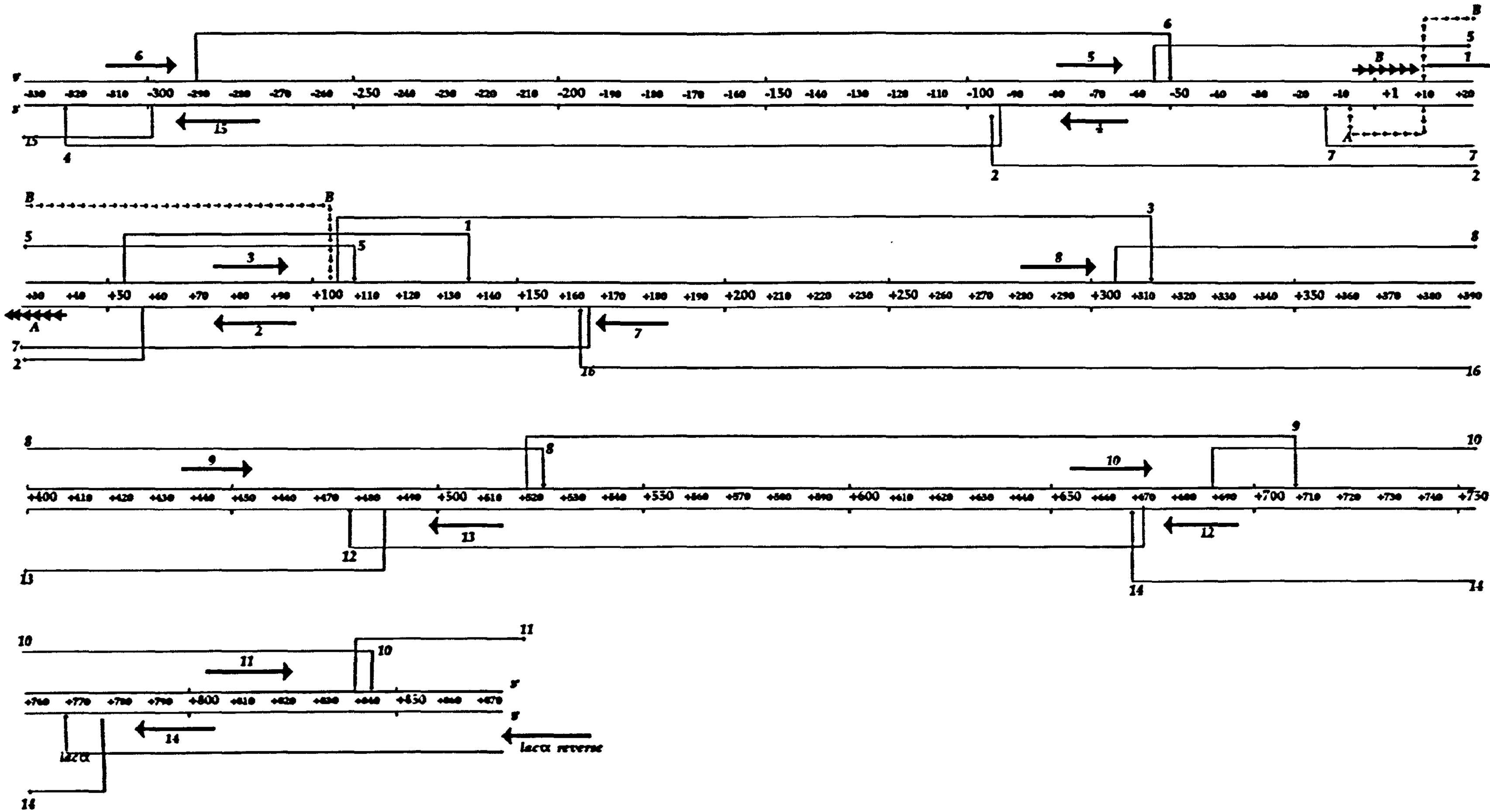


Figure 5. 4. Order of Sequencing

5. 3. Results and Discussion

5. 3. 1. Sequencing Strategy and Reactions

The sequence obtained using the first degenerate primer, primer A, and reverse transcriptase was sufficient to enable the deduction of a second primer despite the subsequent observation that primer A was incorrect in 2 out of its 17 bases (**Figure 5.2**).

Figure 5. 2.

Sequence of Primer A Compared to the Correct Nucleotide Sequence

Primer A	5' TAC CAG AAG CTT TTG CG 3'
Correct Sequence	5' TAC CAC AAG CTT TTA CG 3'

This strategy enabled the sequencing of *cpe* without prior mapping of the pLW1 and provided a rapid confirmation that the conceptual gene product was a protein with the same N-terminal amino acid sequence as the enterotoxin molecule. However it was a mistake not to undertake the simultaneous mapping of the insert once the sequencing was underway. This would have shown that *cpe* backed up to the vector so sequencing from the M13 *lac*α reverse primer could have been initiated. This way the DNA sequence of the cloned *cpe* fragment would have been elucidated more rapidly. In addition to this the realisation that the C-terminal coding regions and 3' untranslated sequences were missing (**Figure 5.6.**) would have been made earlier.

Very little of the sequencing was problem free. Most difficulties were related to the low G+C content (mol% G+C 24-27 (T_m)) of the *C. perfringens* DNA. This meant that the estimated dissociation temperatures for many of the desired primer/template hybrids were very low resulting in a high probability of mispairing of the primers with the template. In order to avoid this type of problem great care was taken over the design of each primer taking account of possible secondary structure formation, misalignment, multiple priming sites etc.. It quickly became apparent that the quality of the oligonucleotide primers was vital for success because the resynthesis of primers enabled the elucidation of previously illegible sequences. The high A-T content, especially in the upstream region (A+T =83%) and the presence of many repeated sequences (**Figure 5.5.**), meant the template was capable of adopting a high secondary structure. This probably explains why even the T7 DNA polymerase had problems with some sequences. For example only a very limited sequence (≅ 30 bp) was obtained from the upstream primer 15,

Figure 5. 5.
DNA Sequence of the *cpe* Upstream Region

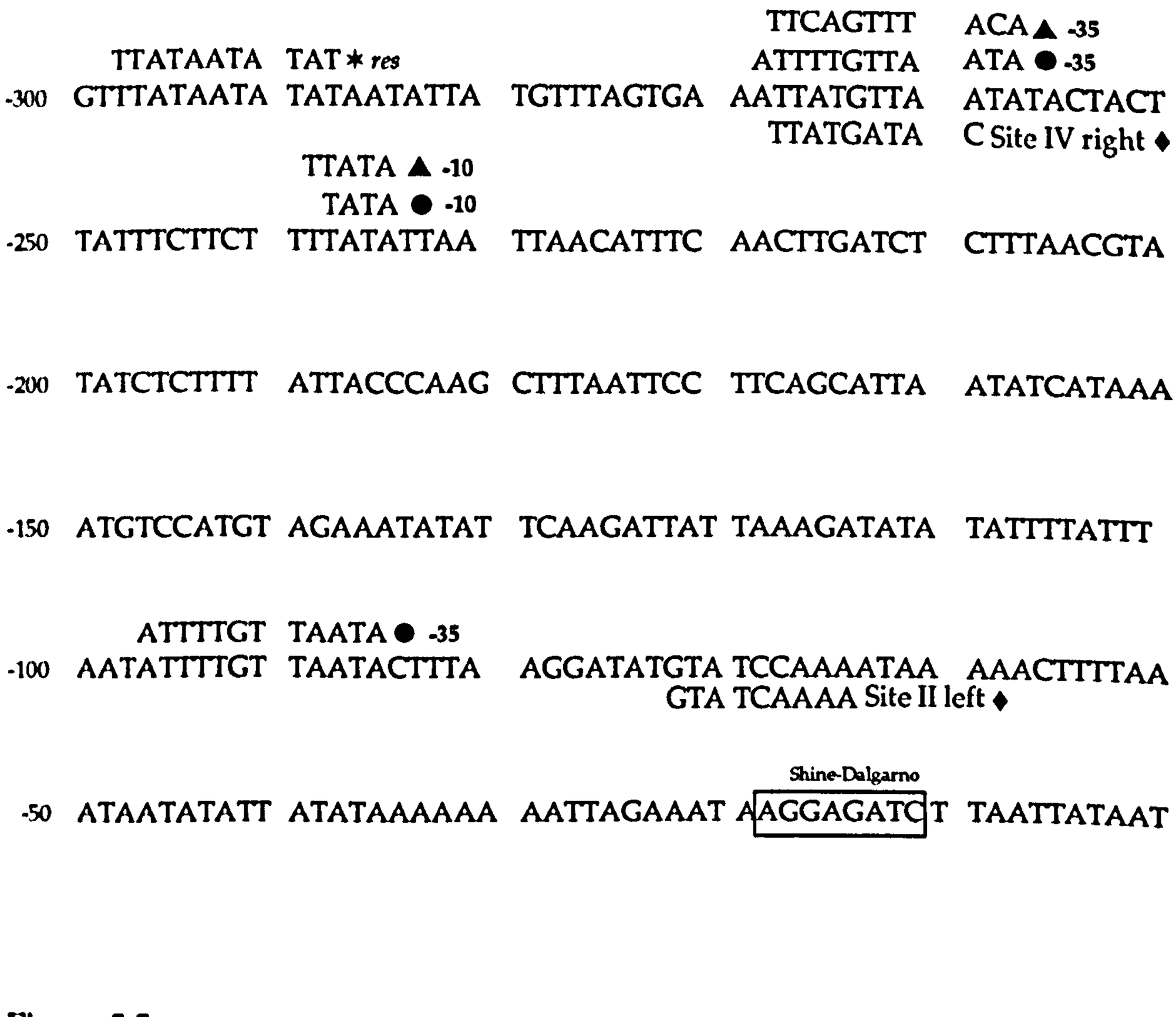


Figure 5.5.

The DNA sequence of the first 300 bp 5' to the *cpe* ATG translation initiation codon. A consensus Shine-Dalgarno sequence is boxed. Also shown are regions with significant homolgy to the putative -10 and -35 promoter sequences, found upstream of the *C. tetani* toxin (●) and the *B. cereus* penicillinase (▲) genes. (✱) is the cross over point in site I of the Tn*3* *res* region. (♦) are arms of the resolvase binding sites in pIP404.

despite resynthesis of the primer, further cleaning of the DNA template and the use of the chase step in the T7 protocol.

5. 3. 2. DNA Sequence of the Enterotoxin Gene

5. 3. 2. 1. Flanking Regions

300 bp of DNA 5' to the *cpe* coding region were sequenced from the pLW1 insert. A Shine-Dalgarno consensus sequence [*Shine & Dalgarno*, 1975] was identified 11 bases upstream of the ATG translation initiation codon and found to be identical to the one found 14 bases 5' to the *C. tetani* tetanus toxin translational start codon [*Eisel et al.*, 1986]. Other regions with significant homology to the putative transcriptional control elements of the tetanus toxin gene were located at -265 bp, -238 bp and -97 bp (the A of the ATG translation initiation codon is designated +1) (**Figure 5.5.**). Bases -238 → -235 are homologous to the putative -10 sequence, whereas sequences -269 → -258 and -97 → -76 are direct repeats of the candidate -35 sequence. These sequences also have similarities to the Pribnow box and -35 control elements of the *B. cereus* penicillinase gene [*Sloma & Gross*, 1983]. Transcript mapping would enable the identification of the transcription start site for *cpe* and would provide the credence necessary to initiate investigations into the possible roles these sequences play in the transcriptional control of *cpe* expression.

The putative -35 sequences also share significant homologies with two resolvase monomer binding sites found in the RES region of the *C. perfringens* bacteriocinogenic plasmid pIP404 [*Garnier et al.*, 1987].

The DNA sequencing downstream of the *cpe* coding region by Van Damme-Jongsten *et al.* [*Van Damme-Jongsten et al.*, 1989] reveals the use of the same translation termination codon TAA in all three reading frames. Unfortunately insufficient sequencing data was published to enable the identification of sequences similar to the *E. coli* Rho-dependent transcription terminators [Review: *Friedman et al.*, 1987]. The mRNA transcript would be able to form the AU rich stem loop, characteristic of Rho-dependent and independent transcription terminators, due to the 17 bp separation of two 11 bp inverted repeat sequences (**Figure 5.7.**).

Interestingly the 5' flanking DNA contains a DNA sequence homologous to the crossover point found in the resolvase binding site I of the *res* region in the Tn*3* subfamily of transposons [*Sherratt*, 1989]. An imperfect, inverted repeat of the same sequence is found with in the coding region [**Figure 5.7.**]. The possible significance of this with respect to earlier observations made during the isolation of pLW1 (Chapter 4) and to the intrastrain variation in *cpe* expression are discussed in chapter 10.

Figure 5. 6.

Base Sequence of the Coding DNA Strand with Deduced Amino Acid Sequence of the Cloned Enterotoxin Gene

				5					10
Met	Leu	Ser	Asn	Asn	Leu	Asn	Pro	Met	Val
ATG	CTT	AGT	AAC	AAT	TTA	AAT	CCA	ATG	GTG
				15					20
Phe	Glu	Asn	Ala	Lys	Glu	Val	Phe	Leu	Ile
TTC	GAA	AAT	GCT	AAA	GAA	GTA	TTT	CTT	ATT
				25					30
Ser	Glu	Asp	Leu	Lys	Thr	Pro	Ile	Asn	Ile
TCT	GAG	GAT	TTA	AAA	ACA	CCA	ATT	AAT	ATT
				35					40
Thr	Asn	Ser	Asn	Ser	Asn	Leu	Ser	Asp	Gly
ACA	AAC	TCT	AAC	TCA	AAT	TTA	AGT	GAT	GGA
				45					50
Leu	Tyr	Val	Ile	Asp	Lys	Gly	Asp	Gly	Trp
TTA	TAT	GTA	ATA	GAT	AAA	GGA	GAT	GGT	TGG
				55					60
Ile	Leu	Gly	Glu	Pro	Ser	Val	Val	Ser	Ser
ATA	TTA	GGG	GAA	CCC	TCA	GTA	GTT	TCA	AGT
				65					70
Gln	Ile	Leu	Asn	Pro	Asn	Glu	Thr	Gly	Thr
CAA	ATT	CTT	AAT	CCT	AAT	GAA	ACA	GGT	ACC
				75					80
Phe	Ser	Gln	Ser	Leu	Thr	Lys	Ser	Lys	Glu
TTT	AGC	CAA	TCA	TTA	ACT	AAA	TCT	AAA	GAA
				85					90
Val	Ser	Ile	Asn	Val	Asn	Phe	Ser	Val	Gly
GTA	TCT	ATA	AAT	GTA	AAT	TTT	TCA	GTT	GGA
				95					100
Phe	Thr	Ser	Glu	Phe	Ile	Gln	Ala	Ser	Val
TTT	ACT	TCT	GAA	TTT	ATA	CAA	GCA	TCT	GTA
				105					110
Glu	Tyr	Gly	Phe	Gly	Ile	Thr	Ile	Gly	Glu
GAA	TAT	GGA	TTT	GGA	ATA	ACT	ATA	GGA	GAA
				115					120
Gln	Asn	Thr	Ile	Glu	Arg	Ser	Val	Ser	Thr
CAA	AAT	ACA	ATA	GAA	AGA	TCT	GTA	TCT	ACA
				125					130
Thr	Ala	Gly	Pro	Asn	Glu	Tyr	Val	Tyr	Tyr
ACT	GCT	GGT	CCA	AAT	GAA	TAT	GTA	TAT	TAT

				135					140
Lys	Val	Tyr	Ala	Thr	Tyr	Arg	Lys	Tyr	Gln
AAG	GTT	TAT	GCA	ACT	TAT	AGA	AAG	TAT	CAA
				145					150
Ala	Ile	Arg	Ile	Ser	His	Gly	Asn	Ile	Ser
GCT	ATT	AGA	ATT	TCT	CAT	GGT	AAT	ATC	TCT
				155					160
Asp	Asp	Gly	Ser	Ile	Tyr	Lys	Leu	Thr	Gly
GAT	GAT	GGA	TCA	ATT	TAT	AAA	TTA	ACA	GGA
				165					170
Ile	Trp	Leu	Ser	Lys	Thr	Ser	Ala	Asp	Ser
ATA	TGG	CTT	AGT	AAA	ACA	TCT	GCA	GAT	AGC
				175					180
Leu	Gly	Asn	Ile	Asp	Gln	Gly	Ser	Leu	Ile
TTA	GGA	AAT	ATT	GAT	CAA	GGT	TCA	TTA	ATT
				185					190
Glu	Thr	Gly	Glu	Arg	Cys	Val	Leu	Thr	Val
GAA	ACT	GGT	GAA	AGA	TGT	GTT	TTA	ACA	GTT
				195					200
Pro	Ser	Thr	Asp	Ile	Glu	Lys	Glu	Ile	Leu
CCA	TCT	ACA	GAT	ATA	CAA	AAG	GAA	ATC	CTT
				205					210
Asp	Leu	Ala	Ala	Ala	Thr	Glu	Arg	Leu	Asn
GAT	TTA	GCT	GCT	GCT	ACA	GAA	AGA	TTA	AAT
				215					220
Leu	Thr	Asp	Ala	Leu	Asn	Ser	Asn	Pro	Ala
TTA	ACT	GAT	GCA	TTA	AAC	TCA	AAC	CCA	GCT
				225					230
Gly	Asn	Leu	Tyr	Asp	Trp	Arg	Ser	Ser	Asn
GGA	AAT	TTA	TAT	GAT	TGG	CGT	TCT	TCT	AAC
				235					240
Ser	Tyr	Pro	Trp	Thr	Gln	Lys	Leu	Asn	Leu
TCA	TAC	CCT	TGG	ACT	CAA	*AAG*	*CTT*	AAT	TTA
				245					250
His	Leu	Thr	Ile	Thr	Ala	Thr	Gly	Gln	Lys
CAC	TTA	ACA	ATT	ACA	GCT	ACT	GGA	CAA	AAA
				255					260
Tyr	Arg	Ile	Leu	Ala	Ser	Lys	Ile	Val	Asp
TAT	AGA	ATC	TTA	GCT	AGC	AAA	ATT	GTT	GAT
				265					270
Phe	Asn	Ile	Tyr	Ser	Asn	Asn	Phe	Asn	Asn
TTT	ATT	ATT	TAT	TCA	AAT	AAT	TTT	AAT	AAT

				275					280
Leu	Val	Lys	Leu	Glu	Gln	Ser	Leu	Gly	Asp
CTA	GTG	AAA	TTA	GAA	CAG	TCC	TTA	GGT	GAT
				285				289	
Gly	Val	Lys	Asp	His	Tyr	Val	Asp	Ile	
GGA	GTA	AAA	GAT	CAT	TAT	GTT	GAT	ATA*AAGCTT*	

Figure 5.6.
The DNA sequence of the coding strand of the cloned *cpe*, together with the deduced amino acid sequence. All *Hind* III sites within the sequence are highlighted, the last of them identifying the end of the cloned *C. perfringens* DNA in pLW1.

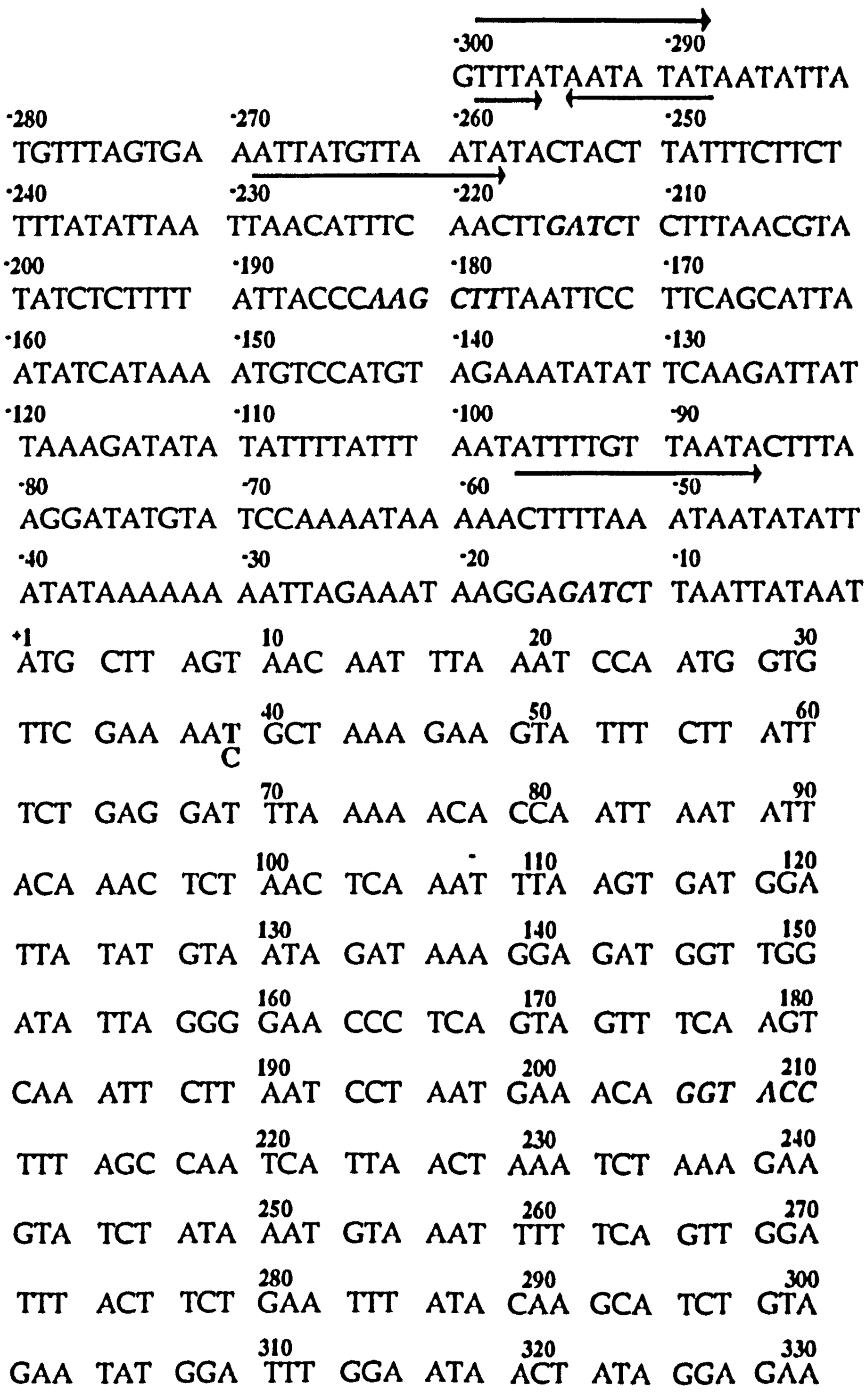
5. 3. 2. 2. Coding Region of the Enterotoxin Gene

Both strands of a 867 bp fragment of pLW1,which codes for 289/321 amino acids of the enterotoxin gene, were sequenced and a theoretical translation product obtained, which was found to correlate well with the published amino acid sequence [*Richardson & Granum*, 1985] (**Figure 5.6.**). The known DNA sequence for the enterotoxin gene is given (**Figure 5.7.**). Conceptual translation of the complete DNA sequence [*Van Damme-Jongsten et al.*, 1989] reveals only one ORF. The amino acid sequence of the resultant protein differed only slightly to the published amino acid sequence for the enterotoxin [*Richardson & Granum*, 1985] (**Figure 5.8.**). The majority of changes occurred on the peptide which Richardson and Granum had identified as one of uncertain amino acid sequence.

The DNA sequence for *cpe* derived from the pLW1 insert differed to the van Damme-Jongsten *et al.* [1989] sequence in three places; a C → T change at position 39 and a C → T change at base 765 are silent but a G → C difference at position 331 results in an amino acid change (Glu to Gln). The sequencing of this codon is shown in **Photograph 5.1.**. It seems that *cpe* may provide support for the 'Neutral Theory of Molecular Evolution' [*Kimura*, 1983] since these are not the only ambiguities observed in the DNA sequence. Van Damme-Jongsten *et al.* [1989] describe several differences between the DNA sequences they derived from two strains of *C. perfringens* typeA, NCTC 8239 and F3686, and Hanna *et al.* [*Hanna et al.*, 1989] published a DNA sequence for part of *cpe* from NCTC 8239 which was identical to the F3686 sequence. One of these base ambiguities, base 459 of the coding region, is adjacent to a copy of the *res* crossover discussed earlier.

Figure 5. 7.

Complete Base Sequence for the Coding DNA Strand, Including the 5' Upstream and 3' Terminal Regions, of *cpe*



```
                                340                             350                             360
CAA  AAT  ACA  ATA  GAA  AGA  TCT  GTA  TCT  ACA
G

                                370                             380                             390
ACT  GCT  GGT  CCA  AAT  GAA  TAT  GTA  TAT  TAT

                                400                             410                             420
AAG  GTT  TAT  GCA  ACT  TAT  AGA  AAG  TAT  CAA

                                430                             440                             450
GCT  ATT  AGA  ATT  TCT  CAT  GGT  AAT  ATC  TCT
                               ------------------------------->

                                460                             470                             480
GAT  GAT  GGA  TCA  ATT  TAT  AAA  TTA  ACA  GGA
           C   ->   ------------>   <------------   <-

                                490                             500                             510
ATA  TGG  CTT  AGT  AAA  ACA  TCT  GCA  GAT  AGC

                                520                             530                             540
TTA  GGA  AAT  ATT  GAT  CAA  GGT  TCA  TTA  ATT

                                550                             560                             570
GAA  ACT  GGT  GAA  AGA  TGT  GTT  TTA  ACA  GTT

                                580                             590                             600
CCA  TCT  ACA  GAT  ATA  GAA  AAG  GAA  ATC  CTT
                                    A

                                610                             620                             630
GAT  TTA  GCT  GCT  GCT  ACA  GAA  AGA  TTA  AAT

                                640                             650                             660
TTA  ACT  GAT  GCA  TTA  AAC  TCA  AAC  CCA  GCT
                                         T

                                670                             680                             690
GGA  AAT  TTA  TAT  GAT  TGG  CGT  TCT  TCT  AAC
T

                                700                             710                             720
TCA  TAC  CCT  TGG  ACT  CAA  AAG  CTT  AAT  TTA

                                730                             740                             750
CAC  TTA  ACA  ATT  ACA  GCT  ACT  GGA  CAA  AAA

                                760                             770                             780
TAT  AGA  ATC  TTA  GCT  AGC  AAA  ATT  GTT  GAT
                               C

                                790                             800                             810
TTT  AAT  ATT  TAT  TCA  AAT  AAT  TTT  AAT  AAT

                                820                             830                             840
CTA  GTG  AAA  TTA  GAA  CAG  TCC  TTA  GGT  GAT

                                850                             860                             870
GGA  GTA  AAA  GAT  CAT  TAT  GTT  GAT  ATA  AGC

                                880                             890                             900
TTA  GAT  GCC  GGA  CAA  TAT  GTT  CTT  GTA  ATG

                                910                             920                             930
AAA  GCT  AAT  TCA  TCA  TAT  AGT  GGA  AAC  TCT

                                940                             950                             960
CAC  CCT  TAT  TCA  ATA  TTA  TTT  CAA  AAA  TTT
```

970 980 990 1000
TAATATTTTA AAATAATATA ATCAAATTAA TTTACAAAAG

1100 1120 1130 1137
ATAGTATGAT AATAATTTAA TATTACATAC TATCTAA

Figure 5.7.
The first base of the ATG methionine translation initiation codon is denoted+1 with all bases 5' to this having a negative prefix. Sites of inter-laboratory or inter-strain ambiguity are highlighted. Recognition sites for the restriction enzymes *Mbo* I, *Hind* III, *Pst* I, *Bgl* II and *Rsa* I are italicised. The repeat sequences discussed in the text are indicated. Data taken from published sequences [*Iwanejko et al.* 1989; *van Damme Jonsten et al.* 1989; *Hanna et al.* 1989] and this publication

Photograph 5. 1.

DNA Sequencing Ladder for the Disputed Base at Position 331

The DNA sequencing for the bases 319 to 342 of the enterotoxin coding region, clearly indicating a C residue at positon 331. The first base in the codon for glutamine, a neutral amino acid. The sequence reported by van Damme-Jongsten *et al.* [1989] has a G in this postion which produces the codon for glutamic acid.

G A T C

342

A
T Ile
A
A
C Thr
A
T
A Asn
A
A
A Gln
C*
A
A Glu
G
A
G Gly
G
A
T Ile
A
T
C Thr

331

319

A

Figure 5. 8.

Revised Amino Acid Sequence of the *C. perfringens* Type A Enterotoxin

									10					
Met	**Leu**	**Ser**	**Asn**	**Asn**	**Leu**	**Asn**	**Pro**	**Met**	**Val**	**Phe**	**Glu**	**Asn**	**Ala**	**Lys**
Met	*Leu*	*Ser*	*Asn*	*Asn*	*Leu*	*Asn*	*Pro*	*Met*	*Val*	*Phe*	*Glu*	*Asn*	*Ala*	*Lys*
				20										30
Glu	**Val**	**Phe**	**Leu**	**Ile**	**Ser**	**Glu**	**Asp**	**Leu**	**Lys**	**Thr**	**Pro**	**Ile**	**Asn**	**Ile**
Glu	*Val*	*Phe*	*Leu*	*Ile*	*Ser*	*Glu*	*Asp*	*Leu*	*Lys*	*Thr*	*Pro*	*Ile*	*Asn*	*Ile*
									40					
Thr	**Asn**	**-----**	**-----**	**-----**	**Ser**	**Asn**	**Ser**	**Asn**	**Leu**	**Ser**	**Asp**	**Gly**	**Leu**	**Tyr**
Thr	*Asn*	*Asn*	*Asn*	*Leu*	*Ser*	*Asn*	*Ser*	*Asn*	*Leu*	*Ser*	*Asp*	*Gly*	*Leu*	*Tyr*
				50										60
Val	**Ile**	**Asp**	**Lys**	**Trp**	**Asp**	**Gly**	**Leu**	**Ile**	**Leu**	**Gly**	**Glu**	**Pro**	**Ser**	**Val**
Val	*Ile*	*Asp*	*Lys*	*Gly*	*Asp*	*Gly*	*Leu*	*Ile*	*Leu*	*Gly*	*Glu*	*Pro*	*Ser*	*Val*
									70					
Val	**Ser**	**Ser**	**Gln**	**Ile**	**Leu**	**Asn**	**Pro**	**Asn**	**Glu**	**Thr**	**Gly**	**Thr**	**Phe**	**Ser**
Leu	*Ser*	*Ser*	*Gln*	*Ile*	*Leu*	*Asn*	*Pro*	*Asn*	*Glu*	*Thr*	*Gly*	*Thr*	*Phe*	*Ser*
				80							(Asn	Phe	Ser)	90
Gln	**Ser**	**Leu**	**Thr**	**Lys**	**Ser**	**Lys**	**Glu**	**Val**	**Ser**	**Ile**	**Asn**	**Val**	**▼Val**	**Gly**
Gln	*Ser*	*Leu*	*Thr*	*Lys*	*Ser*	*Lys*	*Glu*	*Val*	*Ser*	*Ile*	*Asn*	*(Val*	*Val*	*Gly*
									100					
(Phe	Thr	Ser	Glu)		(Gly	Phe	Gly	Ile	Thr	Ile	Gly	Glu	Gln/Glu	Asn)
▼Phe	**Ile**	**Glu**	**Ala**	**Ser**	**Val**	**Glu**	**Tyr**	**-----**	**-----**	**-▼-**	**-----**	**-----**	**Thr**	**Ile**
Phe;	*Ile*	*Gln*	*Ala*	*Ser*	*Val*	*Glu*	*Tyr;*	*Phe*	*Thr*	*Ser*	*Glu*	*Phe)*	*Thr*	*Ile*
				110										120
Glu	**Arg**	**Ser**	**Val**	**Ser**	**Thr**	**Thr**	**Ala**	**Gly**	**Pro**	**Aan**	**Glu**	**Tyr**	**Val**	**Tyr**
Glu	*Arg*	*Ser*	*Val*	*Ser*	*Thr*	*Thr*	*Ala*	*Gly*	*Pro*	*Asn*	*Glu*	*Tyr*	*Val*	*Tyr*
									130					
Tyr	**Lys**	**Val**	**Tyr**	**Ala**	**Thr**	**Tyr**	**Arg**	**Lys**	**Tyr**	**Gln**	**Ala**	**Ile**	**Arg**	**Ile**
Tyr	*Lys*	*Val*	*Tyr*	*Ala*	*Thr*	*Tyr*	*Arg*	*Lys*	*Tyr*	*Gln*	*Ala*	*Ile*	*Arg*	*Ile*
				140										150
Ser	**His**	**Gly**	**Asn**	**Ile**	**Ser**	**Asp**	**Asp**	**Gly**	**Ser**	**Ile**	**Tyr**	**Lys**	**Leu**	**Thr**
Ser	*His*	*Gly*	*Asn*	*Ile*	*Ser*	*Asp*	*Asp*	*Gly*	*Ser*	*Ile*	*Tyr*	*Lys*	*Leu*	*Thr*
									160					
Gly	**Ile**	**Trp**	**Leu**	**Ser**	**Lys**	**Thr**	**Ser**	**Ala**	**Asp**	**Ser**	**Leu**	**Gly**	**Asn**	**Ile**
Gly	*Ile*	*Trp*	*Leu*	*Ser*	*Lys*	*Thr*	*Ser*	*Ala*	*Asp*	*Ser*	*Leu*	*Gly*	*Asn*	*Ile*
				170										180
Asp	**Gln**	**Gly**	**Ser**	**Leu**	**Ile**	**Glu**	**Thr**	**Gly**	**Glu**	**Arg**	**Cys**	**Val**	**Leu**	**Thr**
Asp	*Gln*	*Gly*	*Ser*	*Leu*	*Ile*	*Glu*	*Thr*	*Gly*	*Glu*	*Arg*	*Cys*	*Val*	*Leu*	*Thr*
									190					
Val	**Pro**	**Ser**	**Thr**	**Asp**	**Ile**	**Glu**	**Lys**	**Glu**	**Ile**	**Leu**	**Asp**	**Leu**	**Ala**	**Ala**
Val	*Pro*	*Ser*	*Thr*	*Asp*	*Ile*	*Glu*	*Lys*	*Glu*	*Ile*	*Leu*	*Asp*	*Leu*	*Ala*	*Ala*
				200										210
Ala	**Thr**	**Glu**	**Arg**	**Leu**	**Asn**	**Leu**	**Thr**	**Asp**	**Ala**	**Leu**	**Asn**	**Ser**	**Asn**	**Pro**
Ala	*Thr*	*Glu*	*Arg*	*Leu*	*Asn*	*Leu*	*Thr*	*Asp*	*Ala*	*Leu*	*Asn*	*Ser*	*Asn*	*Pro*
									220					
Ala	**Gly**	**Asn**	**Leu**	**Tyr**	**Asp**	**Trp**	**Arg**	**Ser**	**Ser**	**Asn**	**Ser**	**Tyr**	**Pro**	**Trp**
Ala	*Gly*	*Asn*	*Leu*	*Tyr*	*Asp*	*Tyr*	*Arg*	*Ser*	*Ser*	*Asn*	*Ser*	*Tyr*	*Pro*	*Trp*

				230										240
Thr	**Gln**	**Lys**	**Leu**	**Asn**	**Leu**	**His**	**Leu**	**Thr**	**Ile**	**Thr**	**Ala**	**Thr**	**Gly**	**Gln**
Thr	*Gln*	*Lys*	*Leu*	*Asn*	*Leu*	*His*	*Leu*	*Thr*	*Ile*	*Thr*	*Ala*	*Thr*	*Gly*	*Gln*

									250					
Lys	**Tyr**	**Arg**	**Ile**	**Leu**	**Ala**	**Ser**	**Lys**	**Ile**	**Val**	**Asp**	**Phe**	**Asn**	**Ile**	**Tyr**
Lys	*Tyr*	*Arg*	*Ile*	*Leu*	*Ala*	*Ser*	*Lys*	*Ile*	*Val*	*Asp*	*Phe*	*Asn*	*Ile*	*Tyr*

				260										270
				(Asn)										
Ser	**Asn**	**Asn**	**Phe**	**▼Asn**	**Leu**	**Val**	**Lys**	**Leu**	**Glu**	**Gln**	**Ser**	**Leu**	**Gly**	**Asp**
Ser	*Asn*	*Asn*	*Phe*	*Asn*	*Leu*	*Val*	*Lys*	*Leu*	*Glu*	*Gln*	*Ser*	*Leu*	*Gly*	*Asp*

									280					
Gly	**Val**	**Lys**	**Asp**	**His**	**Tyr**	**Val**	**Asp**	**Ile**	**Ser**	**Leu**	**Asp**	**Ala**	**Gly**	**Gln**
Gly	*Val*	*Lys*	*Asp*	*His*	*Tyr*	*Val*	*Asp*	*Leu*	*Ser*	*Leu*	*Asp*	*Ala*	*Gly*	*Gln*

				290										300
														(Ser His)
Tyr	**Val**	**Leu**	**Val**	**Met**	**Lys**	**Ala**	**Asn**	**Ser**	**Ser**	**Tyr**	**Ser**	**Gly**	**Asn**	**-▼-**
Tyr	*Val*	*Leu*	*Val*	*Met*	*Lys*	*Ala*	*Asn*	*Ser*	*Ser*	*Tyr*	*Ser*	*Gly*	*Asn*	*Tyr*

								309
Pro	**Tyr**	**Ser**	**Ile**	**Leu**	**Phe**	**Gln**	**Lys**	**Phe**
Pro	*Tyr*	*Ser*	*Ile*	*Leu*	*Phe*	*Gln*	*Lys*	*Phe*

Figure 5. 8.

The amino acid sequence derived by conceptual tranlation of the *cpe* DNA sequence [*Iwanejko et al.* 1989; *van Damme Jonsten et al.* 1989; *Hanna et al.* 1989; and this publication] is shown, in bold type, above the amino sequence obtained by peptide analysis of the purified enterotoxin molecule [*Richardson & Granum.* 1985]. — indicates amino acids missing from the conceptual sequence. Additional amino acids are bracketed and ▼ indicates the site of insertion. One of the sites of ambiguity that exists between the two *cpe* DNA sequences would result in the insertion of either glutamine or glutamic acid, both amino acids are shown but in a different type setting to the rest of the sequence.

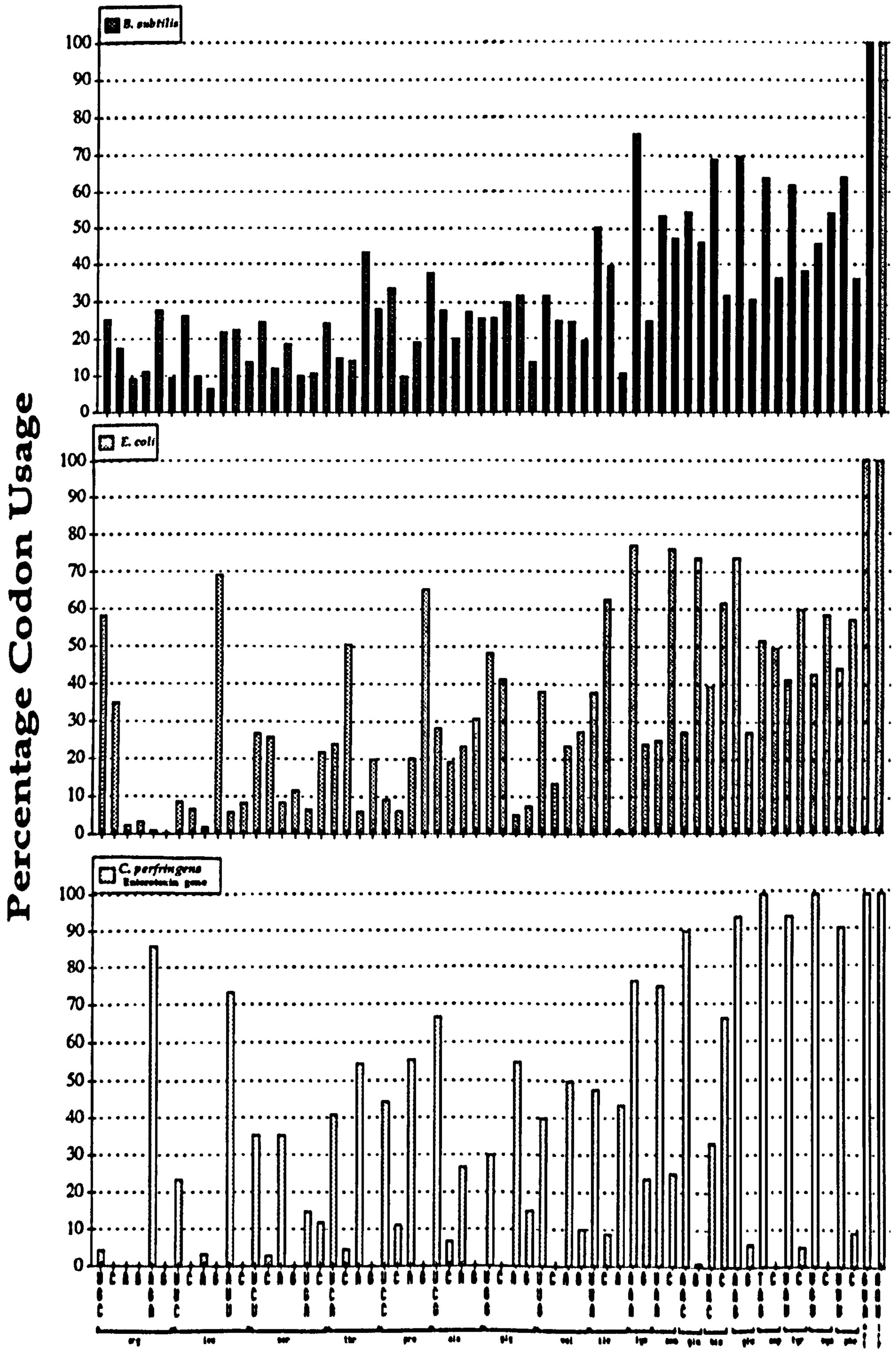
5. 3. 2. 3. Base Composition and Codon Usage

The high A-T content (83%) of the upstream region, especially at the -10 sequence, is in accord with the theoretical requirement for a sequence with a low melting temperature to facilitate the conversion of the initial "closed" binary complex to the "open" binary complex [*Glass*, 1982]. It also complies with the integration site specifications for the mobile genetic element IS1 [*Berg*, 1990]. The strings of As and Ts are also characteristic of bent DNA, the best example of which is the circular kinetoplast DNA of *Crithidia fasciculata* [*Kitchen et al.*, 1986].

The codon biases shown by *cpe* (**Figure 5.9.**) are in accord with those for other clostridial species [*Young et al.*, 1989]. The considerable codon bias, much more than in *E. coli*, should be taken account of during the design of degenerate oligonucleotide probes and primers. Apart from the alanine codon GCU (66.07%) *cpe* did not show preferences for any of the codons thought to correlate with high gene expressivity i.e. C in the third base position when the first two bases are both A or U and U in the third place when the first two bases are G or C [*Gouy & Gautier*, 1982; *Grosjean & Fiers*, 1982].

Figure 5. 9.

cpe Codon Usage Compared to Codon Biases in *E. coli* and *B. subtilis*



CHAPTER 6

PROBING THE PLASMIDS OF FOOD POISONING ASSOCIATED *C. PERFRINGENS* ISOLATES FOR THE ENTEROTOXIN GENE

6. 1. Introduction

Plasmid analyses of clostridia are in their infancy compared to other bacteria, especially *Staphylococcus aureus* [*Archer et al.*, 1982; *Baumergartner et al.*, 1984; *Dodd et al.*, 1987; *Dodd et al.*, 1988], however one study of *C. botulinum* and related organisms revealed that strains from a variety of different geographical locations frequently possessed plasmids that were similar in size and restriction pattern [*Strom et al.*, 1984]. More detailed investigations found that for *C. botulinum* type G strains, production of neurotoxin and bacteriocin was concomitant with the possession of an 81MDa plasmid [*Eklund et al.*, 1988]. This observation could be explained if either the plasmid encoded the neurotoxin structural gene or if the plasmid was a functional *trans*-acting regulator of the neurotoxin gene. In contrast to the *C. botulinum* study, a similar plasmid analysis of 62 epidemiologically unrelated *C. perfringens* isolates failed to find any similarities between strains [*Mahony et al.*, 1987]. However a plasmid profiling study of *C. perfringens* isolates [*Phillips Jones et al.*, 1989] observed that a high proportion of food poisoning associated isolates contained plasmids (61%; 21 isolates). Also a plasmid band which migrated to the 6.0-7.0 kbp mark, the size of the only *C. perfringens* transposable elements discovered so far, Tn*4451* and Tn*4452* [*Abraham and Rood*, 1987], was found to occur significantly more often in these strains, 71% (15/21) compared to non-food poisoning associated isolates 19% (8/43). To ascertain if the enterotoxin gene was plasmid located, bearing in mind the hypothesis concerning the *C. botulinum* 81MDa plasmid, the plasmids from most of the food poisoning associated strains described by Phillips Jones *et al.* [1989] were probed with a *cpe* derived oligonucleotide probe. It was thought unlikely that the plasmids would hybridize to the probe since the enterotoxin gene had been cloned from strain NCTC 8239, a producer of high levels of enterotoxin but apparently devoid of plasmids. However this high level of enterotoxin synthesis could be the result of altered transcriptional regulation of *cpe*, due to plasmid integration into the chromosome.

6. 2. Materials and Methods

6. 2. 1. Strains

Only those strains that had been deposited in the National Collection of Type Cultures were known to be toxin type A. No definite information regarding the enterotoxin synthesising abilities of any of the strains, except NCTC 8239, was available, however all of the nineteen strains of *C. perfringens* used in this experiment had been isolated during, and assumed to be the causative agents of, food poisoning incidents. All the known strain characteristics are presented in **Table 6.1.**.

The NCTC and F strains were a gift from Dr. J. L. Smart, Institute of Food Research, Bristol Laboratory. The PHL strains were obtained from the Food Hygiene Laboratory, Central Public Health Laboratory, London.

The methods of Hauschild and Hilsheimer [1974], (Appendix I), were used to confirm that all the strains were Gram positive, non-motile rods that reduced nitrate, liquefied gelatin and fermented lactose.

Strains were maintained in Cooked Meat Media [OXOID], initially at 4°C, but later at room temperature on the advice of a colleague.

6. 2. 2. Plasmid Isolation and Gel Electrophoresis

The study organisms were inoculated into 2 ml BHI [OXOID] and grown up overnight in an anaerobic jar at 37°C. All 2 ml of overnight culture was utilised for the plasmid extraction procedure, a modified version of the Mahony [*Mahony et al.*, 1986] lysozyme-sarcosyl method (Appendix II). No attempts to eliminate open-circle or linear forms were made before loading the entire extraction (50 µl including loading dye) onto an 0.8% (w/v) Tris-acetate agarose gel (Appendix III).

A *Hin*d III digest (Appendix III) of bacteriophage lambda (λ) was loaded as a size marker and to provide a negative control for the hybridization experiment. Undigested chromosomal DNA from *C. perfringens* NCTC 8239 (the donor strain for the cloned enterotoxin gene, described below) and *Bacillus subtilis* MI112, were positive and negative controls, respectively. A *Hin*d III digest of pLW1, the *E. coli* plasmid vector which contained the cloned *cpe* [*Iwanejko et al.*, 1989], was used as an additional positive control. The plasmids were separated in the gel by electrophoresis overnight (16-18 hours) at 30 V (60mA)(Appendix III).

6. 2. 3. Southern Blotting and DNA Hybridization

DNA was transferred from the agarose gel onto BioTrace™ nylon membranes (Gelman) using a VACU-AID system (a Hybaid™ Southern blotting apparatus) according to the manufacturers instructions. Prehybridization and hybridization were as prescribed for use with the nylon membranes [*Gelman Sciences Inc*, 1987]. Low stringency post hybridization washes (40 ml min^{-1} for 5 minutes) were performed using a Hybaid™ blot processing system, according to the manufacturers specifications.

The *cpe* probe was a 36 base pair oligonucleotide made using phosphoramidite chemistry on an automated DNA synthesizer (Milligen Biosearch). The base sequence (**Figure 6.1.**) corresponded to coding strand bases +7 → +42 (amino acids 3 → 14) inclusive, of the cloned and sequenced enterotoxin gene. The probe was 5' end-labelled [*Maniatis et al.*, 1982] with γ-[^{32}P] dATP [Amersham]. Unincorporated nucleotides were removed from the reaction mix by passage through a Sephadex G-25 column [Pharmacia LKB] equlibrated with TE buffer.

The filters were autoradiographed at -70°C, using double sided high performance autoradigraphy film (Amersham Hyperfilm™) with an intensifying screen. The film was developed for 6 minutes with high contrast developer (Ilford) and fixed, after rinsing in water, until clear with Ilford Speed 2600 fixer.

Figure 6. 1.

Sequence of the Oligonucleotide Probe and Corresponding Amino Acid Sequence

5'	AGT	AAC	AAT	TTA	AAT	CCA	ATG	GTG	TTC	GAA	AAT	GCT	3'
	Ser	Asn	Asn	Leu	Asn	Pro	Met	Val	Phe	Glu	Asn	Ala	
	3	4	5	6	7	8	9	10	11	12	13	14	

6. 3. Results and Discussion

6. 3. 1. Purification of *C. perfringens* Plasmid DNA

The very low purity and quantity of plasmid DNA obtained using the method described above (**Photographs 6.1a.** and **6.2b.**) meant that many preparations had to be done before a gel of a suitable quality for Southern blotting was obtained. There were several reasons why these problems were encountered with *C. perfringens*. Degradation of the plasmid DNA by endogenous endonucleases is frequently encountered unless *end* mutants are used [*Sambrook et al.*, 1989]. The phenol extraction step removes endonucleases but there is often a significant time gap between achieving sufficient cell lysis and the addition of phenol during which some DNA degradation occurs, so a suitable compromise has to be reached. There are always difficulties, whatever the bacteria, in achieving sufficient cell lysis to permit plasmid DNA but not chromosomal DNA to escape. This problem is exacerbated when the cells are difficult to lyse, as is the case with many Gram positive bacteria. This problem can usually be overcome because any contaminating, high molecular weight chromosomal DNA can be separated, along with cell debris, from the plasmid DNA by high speed centrifugation. Unfortunately this process also removes high molecular weight plasmid DNA. An added problem with *C. perfringens* chromosomal and probably also plasmid DNA, was that it sheared very easily to give low molecular weight DNA which was not removed by centrifugation (discussed in Appendix II). A scenario which could result in an incomplete plasmid population being the subject of the investigation was not desirable, because the experiment aimed to ascertain whether or not the enterotoxin gene was located on a plasmid, however the presence of a large amount of contaminating chromosomal DNA which co-migrated with the plasmid DNA would have made the results difficult to interpret. The method finally adopted produced the greatest number of plasmid bands with the least amount of contaminating DNA.

6. 3. 2. Southern Blots

Many plasmid preparations were performed before gels were obtained which had all the plasmid bands that had so far been identified for each of the study strains [*Phillips Jones et al.*, 1989] but very little background DNA. These gels were photographed (**Photographs 6.1a.** and **6.2a. Top**) and blotted onto nylon membranes. The method of DNA transfer, the Hybaid™ VACU-AID system, greatly reduced the time required to perform a Southern blot, from overnight to a couple of hours. The transfer was successful because ethidium bromide bands corresponding to the λ *Hin*d III DNA bands in the gels were easily

visualised on the membrane under ultraviolet light.

The hybridization solution contained formamide which enables the hybridization reaction to be carried out at 42°C, well below the estimated melting temperature (T_m) of 94°C (T_m = 4(G+C)+2(A+T)) for the probe and its target sequence, thereby increasing the stability of such hybrids. The non-specific binding of the probe to λ DNA (**Lanes 13 & 12. Photographs 6.1b.** and **6.2b.** respectively) was an expected side effect of the low stringency hybridization conditions employed. Very low stringency post hybridization washes were used to further increase the chances of detecting any hybridization of the probe to plasmid DNA.

Prehybridization of the membranes and post hybridization washes were performed using the Hybaid™ blot processing system. This method was used because it reduced operator exposure to ionizing radiation and allowed the simultaneous processing of several blots. Unfortunately specific hybridization of the probe was often obscured by very large areas of non specific hybridization. The binding was assumed to be non specific because of its 'non band like' shape and because it was in the same position on autoradiographs of two blots that were manipulated together but had different target DNAs. The most likely cause of this problem was thought to be a processing malfunction, probably the presence of an undetected air bubble at the prehybridization stage preventing the blocking of non-specific DNA binding sites on the nylon membrane which then allowed the irreversible binding of the probe during the hybridization step. The fault was unlikely to be a result of the post hybridization washes because the remaining areas of the membrane were almost completely free of the characteristic non-specific binding spots associated with inadequate washing procedures.

6. 3. 3. Hybridization of the *cpe* Oligonucleotide Probe

Probe specificity was demonstrated by the amount of hybridization to the pLW1 *Hin*d III band which contained the probe sequence (**Lanes 12 & 11. Photographs 6.1b.** and **6.2b.**). The aforementioned non-specific binding to the λ *Hin*d III DNA indicated that the stringency of the post hybridization washing regime would not promote the dissociation of any probe/plasmid hybrids.

The unquestionable specificity of the probe coupled with the lack of any demonstrable hybridization of it to the plasmid DNA (**Lanes 1 → 11. Photograph 6.1b** and **Lanes 2 → 10. Photograph 6.2b.**) was convincing evidence that the enterotoxin gene was not located on any of the plasmids isolated from the food poisoning strains tested. However the strains may contain other

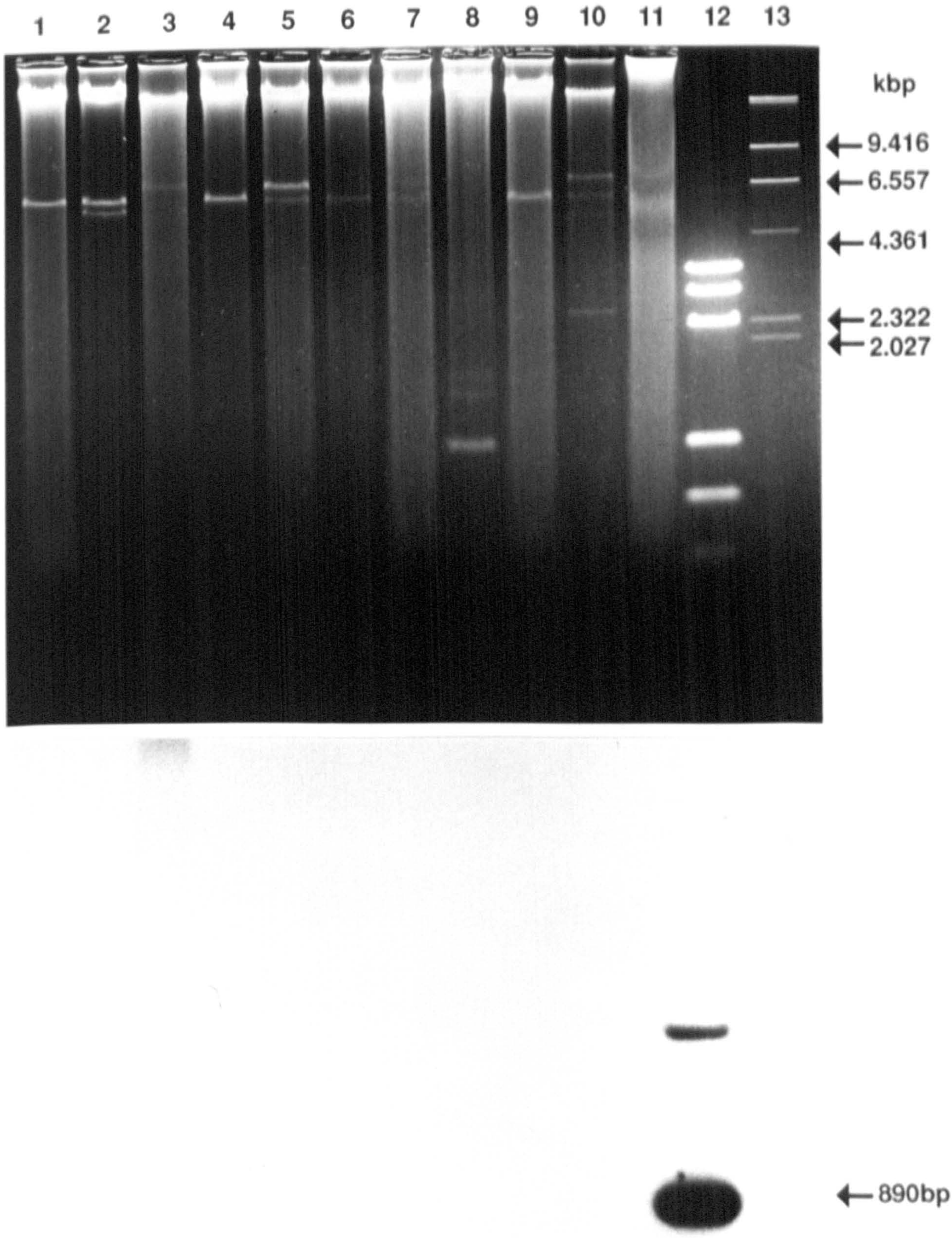
replicons which were not detected in this experiment. Technical limitations would prevent the detection of very large extrachromosomal elements. Also the growth conditions adopted may not be conducive to the mobilization of certain genetic elements such as lysogenic phages, transposons and other episomes.

Although there was an obvious difference between the large amount of probe hybridization with the chromosomal DNA of the enterotoxigenic *C. perfringens* NCTC 8239 compared to the undetectable amount binding with the chromosomal DNA from the non-enterotoxigenic *B. subtilis* MI112 **(Photographs 6.1a & 6.1b.)** the true specificity of the binding was questionable because of the hybridization and washing conditions used. This meant that although the probe had not bound to the plasmid DNA and had hybridized to the chromosomal DNA no justifiable conclusions regarding the chromosomal location of the enterotoxin gene could be made based on these results.

Table 6. 1a.
(Photograph 6. 1a.)
Gel/Blot Order and Description of Strains Used in the Plasmid Hybridization Experiments on Opposite Page

	Strain Designation	Year & Place of Isolation	Heat Resistance; Haemolysis	Serotype Type		Apparent Size (kbp) of Plasmid Bands
1	NCTC 8679	1952 faeces	HR; NK	A (fp)	6	**6.2**
2	NCTC 8235	1951 stew	HR; NK	A (fp)	8	**6.6; 5.9**
3	NCTC 10240	1959 chicken	HR; NK	A (fp)	13	**7.9**
4	NCTC 10239	1961 rissoles	HR; NK	A (fp)	12	**6.0**
5	NCTC 10612	1968 faeces	HR; NK	A (fp)	37	**15.5; 7.5; 7.0**
6	F3417	IFR	HR; H^-	NT (fp)	1	**6.2**
7	F3419	IFR	HR; H^-	NK (fp)	7	**6.6**
8	F1785	IFR	HR; H^+	NK (fp)	38	**1.0**
9	F1415	IFR	HR; H^+	NK (fp)	29	**6.4**
10	F611	IFR	HR; H^-	NK (fp)	13	**7.8; 6.3; 5.4; 2.5**
11	NCTC 8239	1952 salt beef	HR; NK	A (fp)	3	none
12	pLW1 *E. coli* plasmid containing the cloned *cpe* digested with *Hin*d III.					
13	Bacteriophage lambda digested with *Hin*d III.					

NCTC = National Collection of Type Cultures; IFR = Institute of Food Research, Bristol Laboratory; HR = heat resistant; H = haemolysis; A = toxin type A; (fp) = associated with a food poisoning outbreak; NK = not known.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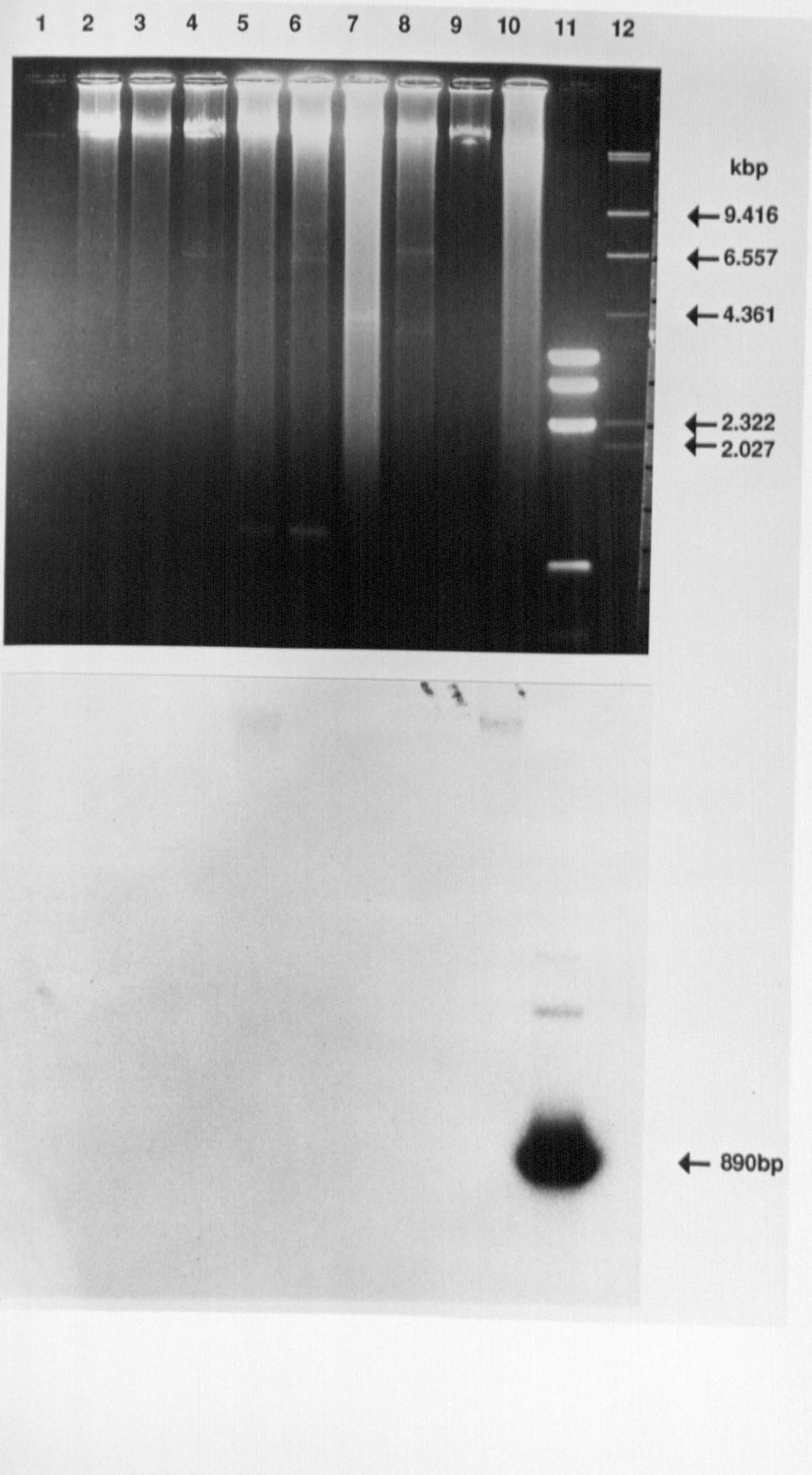
kbp
9.416
6.557
4.361
2.322
2.027
890bp

Table 6. 1b.
(Photograph 6. 1b.)
Gel/Blot Order and Description of Strains Used in the Plasmid Hybridization Experiments on Opposite Page

Strain Designation	Year & Place of Isolation	Heat Resistance; Haemolysis	Toxin Type	Serotype	Apparent Size (kbp) of Plasmid Bands
1	Negative chromosome control. *Bacillus subtilis* MI112 chromosomal DNA				
2	3049 / 86	1986 PHL	NK	NK (fp)	3/4	29.0; **6.5**
3	39 / 86	1986 PHL	NK	NK (fp)	33,62	23.0; 10.0; **6.2**; 3.9
4	73 / 86	1986 PHL	NK	NK (fp)	1	24.5; 9.0; **6.2**
5	1032 / 86	1986 PHL	NK	NK (fp)	52,67	1.0
6	3040 / 86	1986 PHL	NK	NK (fp)	47	8.0; 5.5; >1.0
7	446 / 86	1986 PHL	NK	NK (fp)	8	no plasmids
8	509 / 86	1986 PHL	NK	NK (fp)	3	9.0; **6.6**; 3.8
9	747 / 85	1985 PHL	NK	NK (fp)	3	24.5; 9.0; 5.5
10	Positive chromosome control, same as for gel and blot 6. 1a.				
11	Positive plasmid control, same as for gel and blot 6. 1a.				
12	Size marker and negative control, same as for gel and blot 6. 1a.				

PHL = Food Hygiene Laboratory, Central Public Health Laboratories, London; (fp) = associated with a food poisoning outbreak; NK = not known.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kbp
9.416
6.557
4.361
2.322
2.027
890bp

CHAPTER 7

PROBING THE CHROMOSOMES OF DIFFERENT *C. PERFRINGENS* ISOLATES AND TOXIN TYPES FOR *CPE*

7.1. Introduction

As discussed in Chapter 2 the close association of enterotoxin production with sporulation [*Duncan et. al.*, 1972] led to speculation that the protein was a structural part of the spore coat [*Frieben & Duncan*, 1973]. It was postulated that *cpe* was an essential gene subject to tight regulation of expression which was altered in food poisoning strains [*Granum et al.*, 1984]. If, however, CPE was essential for sporulation it would presumably be required by all strains of *C. perfringens* therefore such deregulated, food poisoning, mutants would occur with equal frequency amongst all the toxin types and this does not appear to happen.

It was therefore surprising when preliminary probing experiments (data not shown) failed to detect the enterotoxin gene in all the strains examined. The subsequent publication of the failure to detect *cpe* in 94% of isolates examined by using colony hybridization [*van Damme-Jongsten et al.*, 1989] seemed to confirm that *cpe* was not an essential gene. It was not however believed that the colony hybridization assays were a suitable means of proving an absence of *cpe* since the inability to hybridize the probe, shown by many of the strains, may have reflected a lack of cell lysis. This type of artifact was avoided by the use of Southern blot analyses of *C. perfringens* DNAs. The presence of test DNAs on the filter was confirmed by visual identification of ethidium bromide stained DNA under UV illumination. Southern blot analysis also enables the simultaneous examination *cpe* architecture.

7.2. Methods

7.2.1. Experiment One

7.2.1.1. Chromosomal DNAs

DNA was isolated from sixteen strains of *C. perfringens* using the modified Okita DNA extraction method (Procedure One. Appendix II). Eight of the strains, including NCTC 8239, were presumed to have been the causative agents of food poisoning outbreaks and all, except F1415, had been categorised in the NCTC as toxin type A. The remaining eight strains had been isolated from lamb carcasses (Appendix I).

Non of the plasmids from the food poisoning strains had hybridized an

enterotoxin gene probe (Chapter 6), the plasmids from the lamb strains had not been probed. All the DNAs were totally digested with *Mbo* I (Appendix III), an enzyme sensitive to adenine methylation. They were then separated on an 0.8% agarose gel with *Hin*d III digested bacteriophage λ and pLW1 DNAs as size markers and negative and positive hybridization controls. The DNA was depurinated and blotted onto nylon membrane, according to the manufacturers instructions [Hybond N. Amersham], using the Hybaid™ blotting apparatus.

7. 2. 1. 2. DNA Probe

pLW1 DNA was digested with *Bgl* II and *Sma* I and separated on a 0.8% preparative agarose gel. A gel slice containing the 520 bp *Bgl* II/*Sma* I fragment (**Figure 7.1.**) was excised and the DNA recovered using the Gene Clean Kit (Appendix III). The fragment was radiolabelled with [α-^{32}P] dCTP using the Multi Primed Labelling Kit [Pharmacia].

7. 2. 1. 3. Hybridization Conditions

Well matched hybrids were expected so hybridization was overnight at 42°C, according to the procedure recommended for nylon membranes with formamide [*Gelman Sciences inc*, 1987] (Appendix V). The filter was then autoradiographed with double sided X-ray film with an intensifying screen at -70°C (Appendix V) (**Photograph 7.1.**).

7. 2. 2. Experiment Two

7. 2. 2. 1. Chromosomal DNAs

DNA was extracted from nine strains of *C. perfringens* strains using the GES method (Appendix II). Five of the strains were associated with food poisoning incidents and four of them had been probed in the earlier experiment. One of the strains was toxin type A but enterotoxin negative. Two toxin type Cs and a toxin type D composed the remainder of the test strains.

One sample of each of the DNAs was digested with *Eco* RI whilst a second sample was digested with *Hin*d III. The DNAs were separated on 0.8% agarose gels with bacteriophage λ and pLW1 DNA controls (**Photographs 7.2. and 7.3.**). The DNA was depurinated by submerging the gel in 0.25M HCl for 20 minutes.The gel was rinsed in water then blotted onto nylon membrane [Hybond N+. Amersham] using 0.4 N NaOH as the transfer and DNA fixing buffer.

7. 2. 2. 2. ^{32}P Labelled DNA Probe

pLW1 DNA was digested with *Pst* I and separated on a 0.6% low melting point agarose gel (Appendix III). A gel slice containing the 460 bp *Pst* I fragment (**Figure 7.2.**) was excised and used directly in a multi primed

labelling reaction (Protocol One. Appendix III).

7. 2. 2. 3. Hybridization Conditions

Hybridization was overnight at 68°C with BLOTTO hybridization buffer (Appendix V). The stringency washes were 10 minutes at 68°C with 3 X SSC: 0.1% SDS and 10 minutes at 68°C with 0.1 X SSC: 0.1% SDS. The final rinse was at room temperature in 3 X SSC. Filters were autoradiographed as before **(Photographs 7.2. and 7.3.).**

7. 3. Results and Discussion

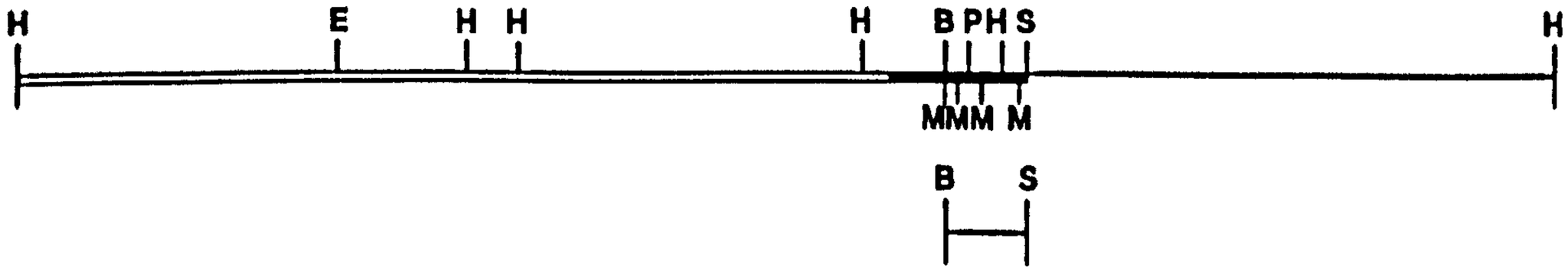
The 520 bp *Bgl* II/*Sma* I *cpe* probe hybridized to *Mbo* I digested DNA from 7/9 food poisoning associated strains but did not detectably bind to the DNAs from any of the lamb carcass *C. perfringens* isolates. In the later experiment *C. perfringens* NCTC 10239 and NCTC 10613 DNAs surprisingly did not hybridize the 460 bp *Pst* I *cpe* probe. Improved DNA extraction, hybridization and probe labelling methods would have resulted in increased sensitivities so it was concluded that either the strains had been mixed up or that subculturing had resulted in the loss of the enterotoxin gene from these strains. Support for the later scenario came from the failure of *C. perfringens* NCTC 8449, a strain isolated in 1952 from steamed lamb and presumed to be the cause of a food poisoning incident, to hybridize either of the *cpe* probes.

Evidence in support of a non essential role for CPE came from the observation that *cpe* was only detected in food poisoning associated type A strains. It was not detected in an enterotoxin negative type A strain, two toxin type C strains a type D strain or the eight lamb strains.

No experiments were performed to assess whether a failure to detect *cpe* correlated with poor sporulation frequencies. Presumably van Damme-Jongsten *et al.* [1989] did not address this question either so their claim to have falsified the spore coat hypothesis [*Frieben & Duncan*, 1973] was not warranted.

The architecture of the 3' end of *cpe* did not vary amongst the seven *Mbo* I digested DNAs which hybridized the *Bgl* II/*Sma* I probe. The variation seen for strain NCTC 10239 was probably due to incomplete digestion of the DNA. The size of the hybridizing fragments, 0.3 kbp and 0.5 kbp, are consistent with the probe hybridizing to the 330 bp *Mbo* I fragment that results from cleavage at bases 520 and 850 plus binding to an incompletely sequenced 0.5 kbp fragment contiguous to this. The other fragments that would result from *Mbo* I digestion of *cpe* would be 60 bp and 110 bp, too small to be detected on an 0.8% gel.

Figure 7. 1.
The 530 bp *Bgl* II / *Sma* I Fragment of pLW1 Used as the First Enterotoxin Gene Probe

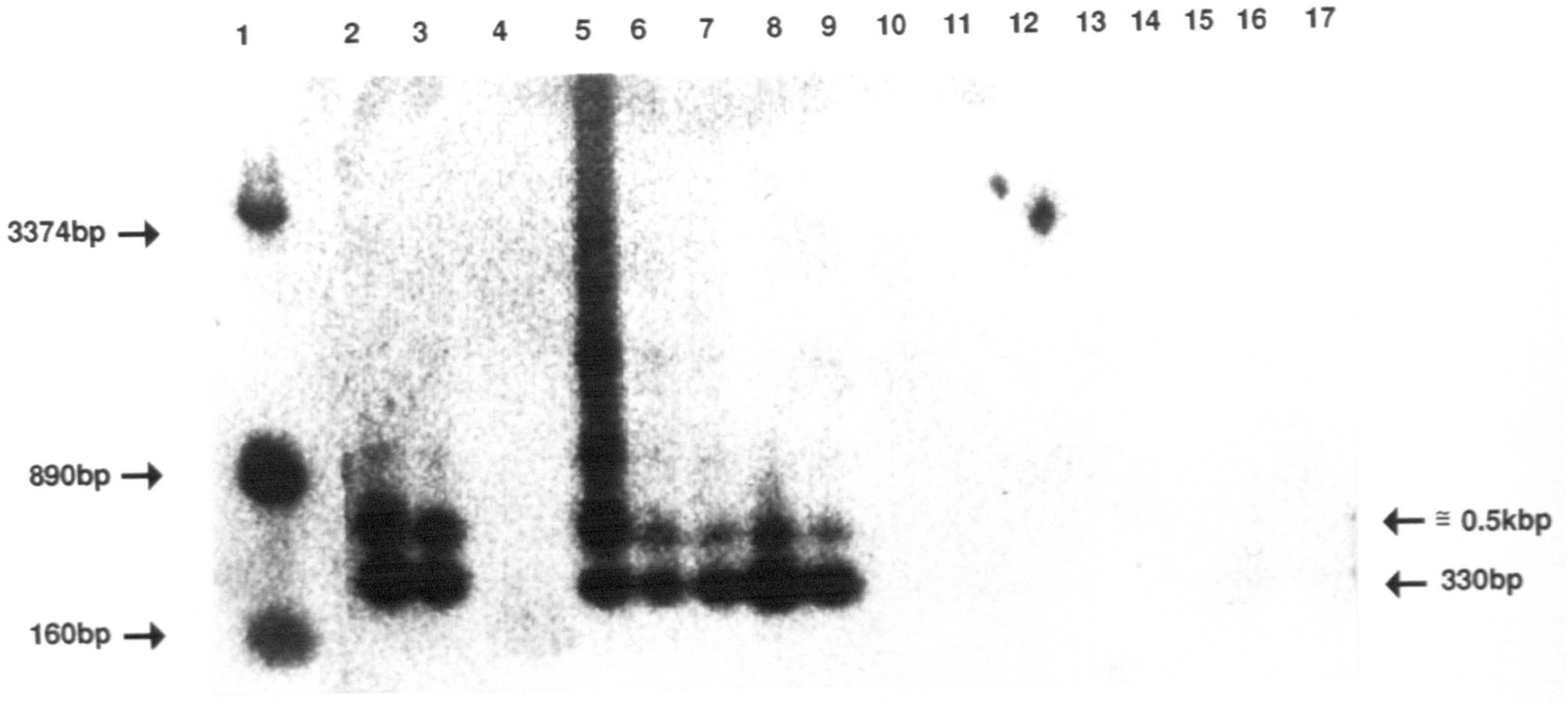


Where H = *Hin*d III, E = *Eco* RI, B = *Bgl* II, P = *Pst* I, S = *Sma* I and M = *Mbo* I.

Photograph 7. 1.

Probing of *Mbo* I Digested DNA of Several *C. perfringens* Isolates for the Enterotoxin Gene

Lane	Description
1	*Hind* III digested pLW1
2	*C. perfringens* NCTC 8239 (no detectable plasmids)
3	*C. perfringens* NCTC 10613 (no detectable plasmids)
4	*C. perfringens* NCTC 8449 (no detectable plasmids)
5	*C. perfringens* F1785 (one plasmid)
6	*C. perfringens* NCTC 10239 (one plasmid)
7	*C. perfringens* NCTC 8235 (two plasmids)
8	*C. perfringens* NCTC 10240 (one plasmid)
9	*C. perfringens* NCTC 8679 (one plasmid)
10	*C. perfringens* L4. 1 (no detectable plasmids)
11	*C. perfringens* L53. 1(no detectable plasmids)
12	*C. perfringens* L60. 2 (no detectable plasmids)
13	*C. perfringens* L5. 4 (four plasmid)
14	*C. perfringens* L99. 4 (seven plasmids)
15	*C. perfringens* L100. 4 (two plasmids)
16	*C. perfringens* L24. 2 (four plasmids)
17	*Hind* III digested bacteriophage λ DNA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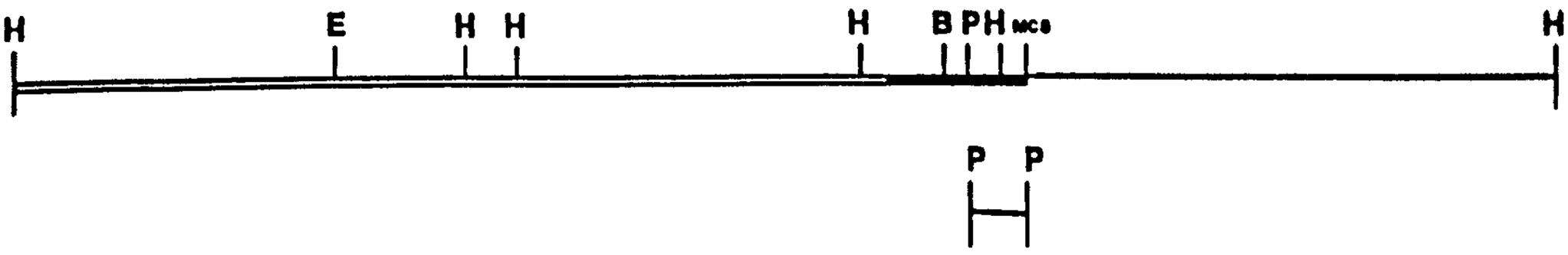
15
16
17
3374bp
890bp
160bp
≅ 0.5kbp
330bp

Figure 7. 2.
The 460 bp *Pst* I Fragment of pLW1 Used as the Second Enterotoxin Gene Probe

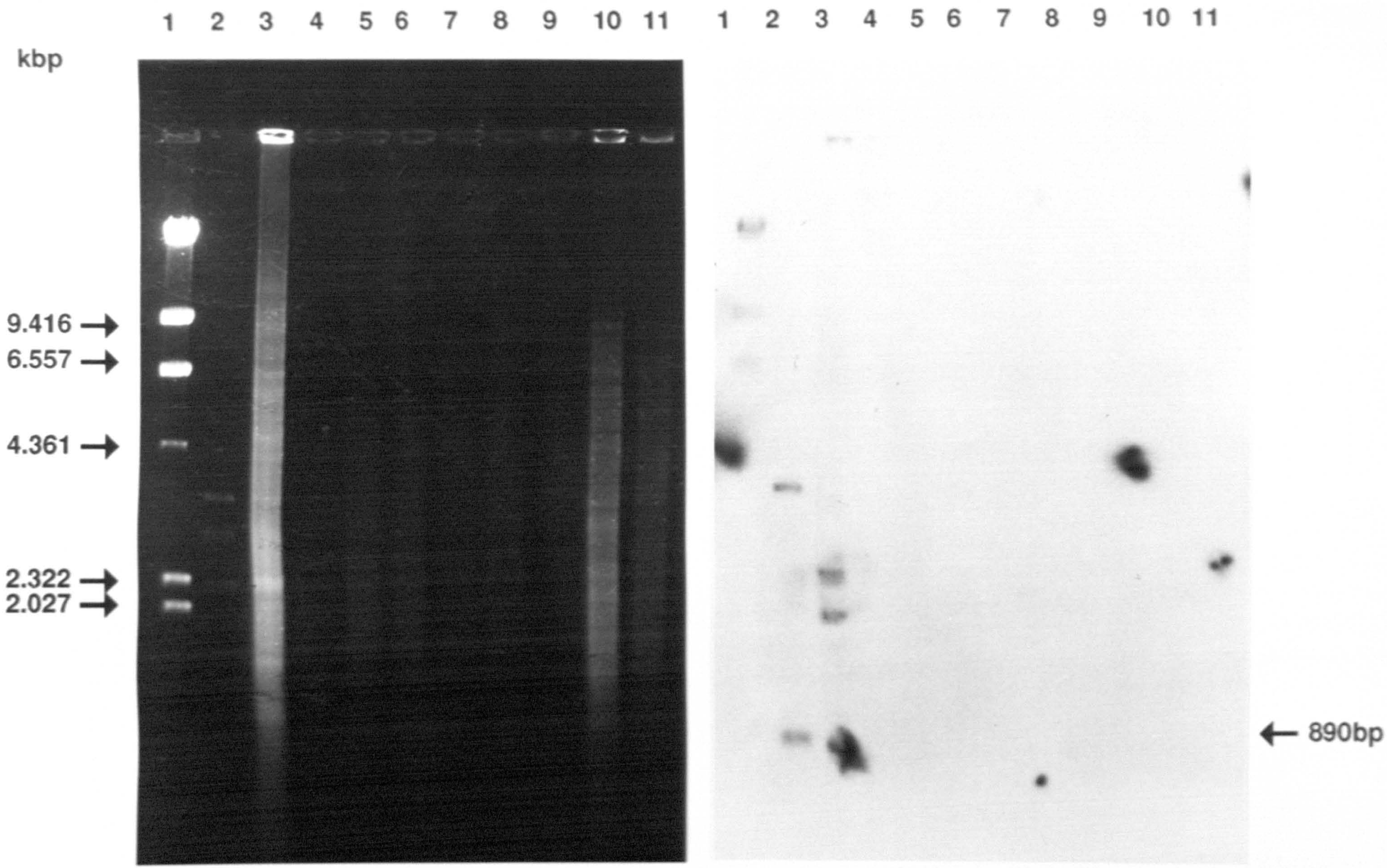


Where H = *Hin*d III, E = *Eco* RI, B = *Bgl* II, P = *Pst* I, mcs = multiple cloning site

Photograph 7. 2.

Probing the *Hin*d III Digested DNA from Different *C. perfringens* Isolates and Toxin Types for the Enterotoxin Gene

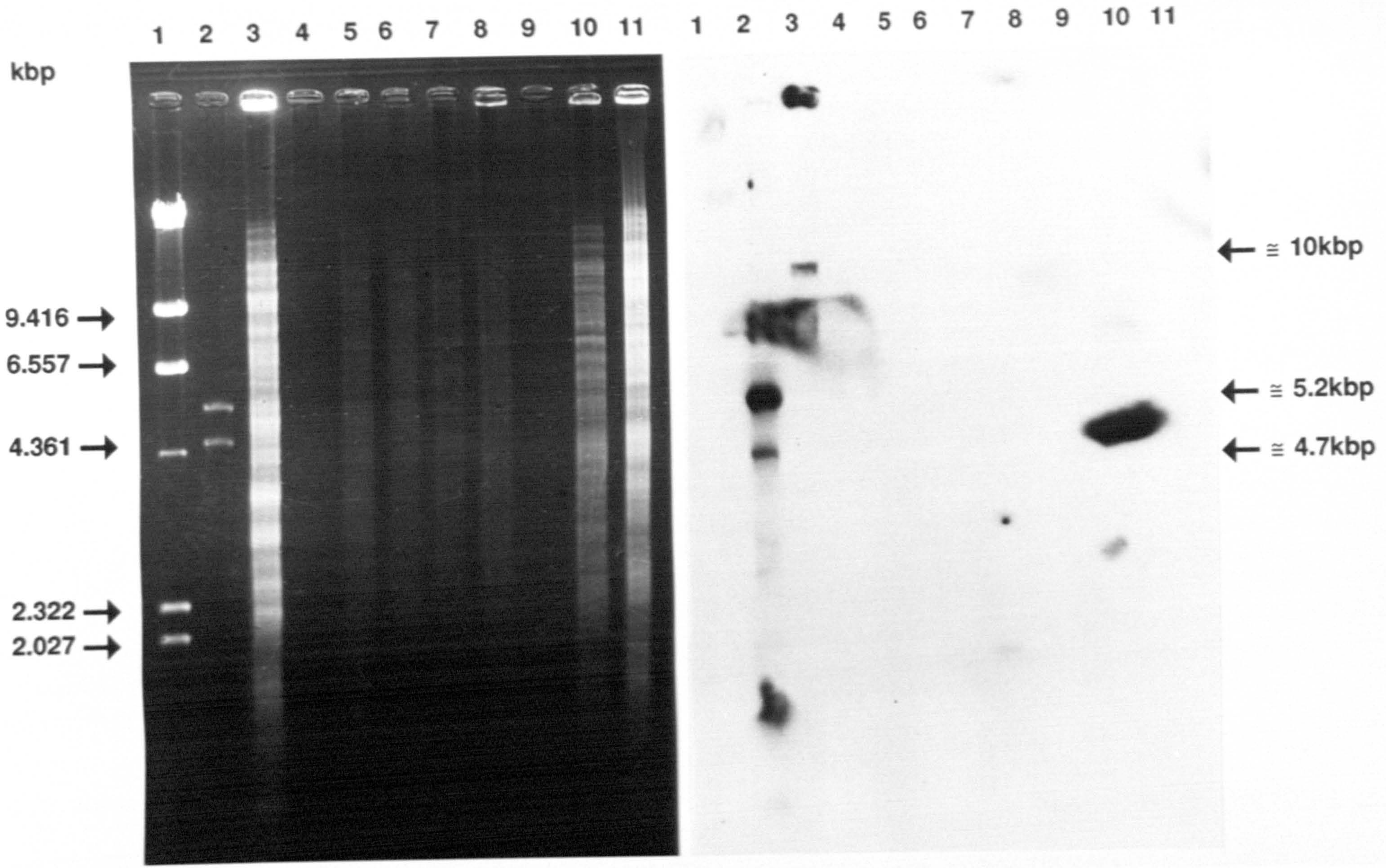
Lane	Description of the DNA
1	*Hin*d III digested bacteriophage λ DNA
2	*Hin*d III digested pLW1
3	*Hin*d III *C. perfringens* NCTC 8239 (has no detectable plasmids)
4	*Hin*d III *C. perfringens* NCTC 8449 (has no detectable plasmids)
5	*Hin*d III *C. perfringens* NCTC 10239 **(has one plasmid ≅ 6.0 kbp)**
6	*Hin*d III *C. perfringens* NCTC 10613 (has no detectable plasmids)
7	*Hin*d III *C. perfringens* type A strain 2498 (plasmid content unknown)
8	*Hin*d III *C. perfringens* type C strain 5381 (plasmid content unknown)
9	*Hin*d III *C. perfringens* type C strain 5383 (plasmid content unknown)
10	*Hin*d III *C. perfringens* type D strain 755 (plasmid content unknown)
11	*Hin*d III *C. perfringens* F1415 **(has one plasmid ≅ 6.4 kbp)**


1 2 3 4 5 6 7 8 9 10 11
kbp
9.416
6.557
4.361
2.322
2.027
1 2 3 4 5 6 7 8 9 10 11
890bp

Photograph 7. 3.

Probing the *Eco* RI Digested DNA from Different *C. perfringens* Isolates and Toxin Types for the Enterotoxin Gene

Lane	Description of the DNA
1	*Hin*d III digested bacteriophage λ DNA
2	*Eco* RI digested pLW1
3	*Eco* RI *C. perfringens* NCTC 8239 (has no detectable plasmids)
4	*Eco* RI *C. perfringens* NCTC 8449 (has no detectable plasmids)
5	*Eco* RI *C. perfringens* NCTC 10239 **(has one plasmid ≅ 6.0 kbp)**
6	*Eco*RI *C. perfringens* NCTC 10613 (has no detectable plasmids)
7	*Eco* RI *C. perfringens* type A strain 2498 (plasmid content unknown)
8	*Eco* RI *C. perfringens* type C strain 5381 (plasmid content unknown)
9	*Eco* RI *C. perfringens* type C strain 5383 (plasmid content unknown)
10	*Eco*RI *C. perfringens* type D strain 755 (plasmid content unknown)
11	*Eco* RI *C. perfringens* F1415 **(has one plasmid ≅ 6.4 kbp)**


1 2 3 4 5 6 7 8 9 10 11
kbp
9.416
6.557
4.361
2.322
2.027
1 2 3 4 5 6 7 8 9 10 11
≅ 10kbp
≅ 5.2kbp
≅ 4.7kbp

CHAPTER 8
RECLONING THE ENTEROTOXIN GENE

8. 1. Introduction to the Recloning Programme

As noted earlier, the sequence of the cloned enterotoxin gene on pLW1 finished short of the end of the predicted coding region (Chapter 5). Preliminary chromosome probing experiments **(Photograph 7.3)**indicated that *cpe* was located on a 10 kbp *Eco* RI fragment. As there was an *Eco* RI site ≅ 3 kbp 5' to *cpe* on pLW1 it was assumed that the 10 kbp fragment would have ≅ 5 kbp of DNA 3' to the end of the cloned region (**Figure 8.2.** Fragment I). A later Southern blot analysis of chromosomal DNA with a *cpe* specific probe confirmed this calculation (**Photograph 8.1.**). The 10 kbp *Eco* RI fragment was therefore a good candidate fragment to enable the cloning of *cpe* in its entirety. The lack of overlapping fragments and the use of *Eco* RI instead of *Hin*d III were the only planned deviations from the original cloning strategy.

Chromosomal DNA was isolated from *C. perfringens* NCTC 8239 as before (Chapter 4). A complete *Eco* RI digest was then shotgun cloned into the multiple cloning site of an *Eco* RI linearised and dephosphorylated (Appendix III) pHG165.

This chapter describes the various *cpe* recloning strategies employed. The order in which they were taken is shown (**Figure 8.1.**) and the reasons for each change are discussed.

Cloning Strategy	*C. perfringens* DNA Fragment	Cloning Vector	*E. coli* Host Strain	Transformation Procedure	DNA Probe	Result
Shotgun	≅ 10 kbp *Eco* RI	pHG165	JM107	$CaCl^T$ Electroporation	N/A	NET
"	"	"	"	"	"	NERT
"	"	pLAFR1	"	"	"	NT
"	≅ 9 kbp *Pst* I	pHG165	"	$CaCl^T$ Electroporation	"	NET
"	"	"	"	"	"	NERT
Enriched	≅ 10 kbp *Eco* RI	pHG165	"	"	"	"
"	"	"	DH5α	$CaCl^M$ Electroporation	"	"
"	"	"	"	"	"	"
"	"	"	XL1-BLUE SURE™	$CaCl^M$	"	"
"	"	"	"	"	1	Probe 1 positive clones but no *cpe*
"	≅ 7 kbp *Eco* RI/*Pst* I	"	"	Electroporation*	2	pLW2 but no 3' *cpe*
"	≅ 7 kbp *Eco* RI/*Pst* I	"	"	"	3	No 3' *cpe*
"	≅ 3 kbp *Hpa* I/*Pst* I	"	"	"	N/A	NERT

Key:
$CaCl^{T \text{ and } M}$ refer to Traditional or Manufacturers methods.
Electroporation*-Transformation efficiencies obtained by electroporation were ≅ to those obtained from $CaCl^M$.
N/A-Not Applicable; NET-Not Enough Transformants; NERT-Not Enough Recombinant Transformants; NT-No Transformants

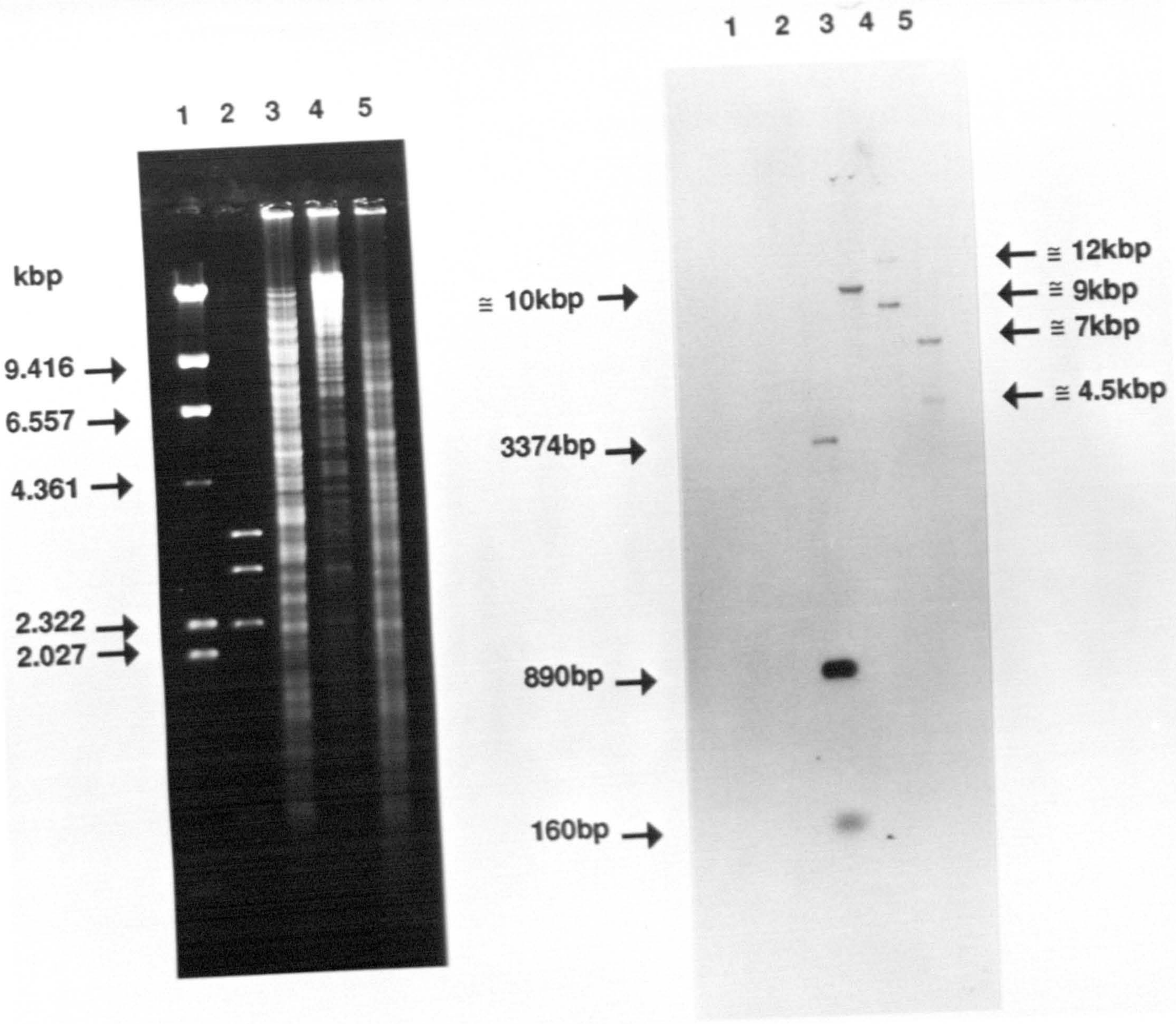
Figure 8. 1. Recloning Experiments

Photograph 8. 1.

Southern Blot Analysis of *C. perfringens* NCTC 8239 Chromosomal DNA with a *cpe* Specific Probe

Lane	Description
1	*Hin*d III digested bacteriophage λ DNA
2	*Hin*d III pLW1 DNA
3	*Eco* RI digested *C. perfringens* NCTC 8239 chromosomal DNA
4	*Pst* I digested *C. perfringens* NCTC 8239 chromosomal DNA
5	*Eco* RI/*Pst* I digested *C. perfringens* NCTC 8239 chromosomal DNA

Agarose gel (left) blotted onto nylon membrane and probed with a *cpe* specific probe (Probe 2. **Figure 8.3.**) and autoradiographed (right).


1 2 3 4 5
kbp
9.416
6.557
4.361
2.322
2.027
1 2 3 4 5
≅ 10kbp
3374bp
890bp
160bp
≅ 12kbp
≅ 9kbp
≅ 7kbp
≅ 4.5kbp

8. 2. Methods and Results

8. 2. 1. Cloning Strategy

A shotgun cloning strategy was originally adopted in order to obtain the near complete *C. perfringens* genomic library needed to ensure a reasonable chance of having the region of interest. It became unnecessary when the size of the required restriction fragment was discovered. Also it was postulated that preliminary attempts to shotgun clone *Eco* RI or *Pst* I digested chromosomal DNAs were failing at the ligation stage. A large proportion of the *C. perfringens* DNA *Eco* RI fragments were very large and therefore liable to be inhibiting the formation of recombinant plasmids at the ligation stage i.e. increasing distance between two ends of a DNA molecule decreases the probability of intramolecular interactions but increases the chance of intermolecular interaction preventing recircularisation. A shotgun approach was therefore abandoned in favour of the following sequence enrichment strategy. Digested chromosomal DNA was electrophoresed in 0.8% preparative agarose gels with *Hin*d III bacteriophage λ DNA size markers. A gel slice deemed to contain DNA of the required size ± 1-2 kbp was excised and the DNA recovered (Appendix III).

8. 2. 2. Transformation Protocols

There were two reasons for trying to increase transformation efficiency by altering the transformation protocols. Firstly, to increase the total numbers of clones containing recombinant plasmids in order to obtain a library sufficiently large to have a reasonable probability of it containing the enterotoxin gene (Chapter 3). Secondly, plasmid DNA over ≅ 15 kbp, a size exceeded by some of the constructs, would not reliably transform cells by traditional transformation procedures.

The unmodified heat shock induced transformation of CaCl treated cells (Appendix IV) regularly gave transformation efficiencies higher than 10^6 transformants μg^{-1} of pHG165 DNA. The modified versions supplied by the manufacturers of ready competent cells gave transformation efficiencies as high as 10^8 cells μg^{-1} of pHG165 DNA. Electroporation induced transformation was purported to give transformation efficiencies up to 10^9-10^{10} transformants μg^{-1} DNA and would also readily induce transformation of cells with large, 25-130 kbp, plasmids [*Dower et al.*, 1988]. High transformation efficiencies, 10^7-10^8 transformants μg^{-1} of pHG165 or pLAFR1 DNAs, were obtained using the recommended protocols (Appendix IV).

8. 2. 3. Host Cells

Host cells were varied to obtain increased transformation efficiencies and to reduce the probability of occurrence of deleterious DNA rearrangements. *E. coli* DH5α, XL1-BLUE and SURE™ (Appendix I) all gave transformation efficiencies in accord with the manufacturers expectations for plasmid vector DNA. Increased numbers of transformants containing recombinant plasmids were obtained with XL1-BLUE cells but a sufficiently large library was only obtained with the SURE™ cells. The newly developed *E. coli* SURE™ were deficient in all known recombination pathways to facilitate the cloning of 'unclonable' DNA.

8. 2. 4. Cloning Vectors

Although the re-introduction of the *rop* gene into pHG165 restored its copy number to one equivalent to pBR322 (Appendix I) there would still have been 15-20 copies per cell. It was proposed that many recombinant plasmids would contain toxin genes (Chapter 2) which could be deleterious to the cell in such high copy number. The low copy number plasmid pLAFR1 [*Friedman et al.*, 1982] (Appendix I), an *Eco* RI cloning vector, was used to test this hypothesis. The number of transformants obtained using the pLAFR1 ligation reaction (pLAFR1 plus *Eco* RI fragments) was identical to the background number obtained due to religation of the vector.

8. 2. 5. *cpe* Probe

A 5.6 kbp *Eco* RI fragment of pLW1 (**Figure 8.3.** Probe 1) was used as an enterotoxin gene probe to enable the use of high stringency hybridization conditions and washes thereby reducing the amount of non specific hybridization associated with colony hybridizations (Appendix V). An unexpectedly high proportion of the Ap^R Lac^- *E. coli* SURE™ colonies, transformed with a ligation reaction between pHG165 and ≅ 10 kbp Eco RI *C. perfringens* chromosomal DNA fragments (see below) hybridized this probe (**Photograph 8.2.** Plate A). The various *Hin*d III digestion patterns observed for mini-preped (Appendix II) plasmid DNA from a random selection of the positive clones (**Photograph 8.2.**) was an indication that the probe had hybridized to several different plasmids. Southern blot analysis of these plasmids with a *cpe* specific probe (**Figure 8.3.** Probe 3) showed that none of them contained the enterotoxin gene (**Photograph 8.2.**). It was thought appropriate to investigate whether the hybridization with the original 5.6 kbp probe was real or experimental error. The probe was broken down into its constituent *Hin*d III fragments and the smallest (**Figure 8.3.** Probe 2) used to probe the plasmids. *Hin*d III digests of the plasmid DNAs obtained in the original cloning experiment (chapter 4) were also probed. All the new

plasmids and pLW1 hybridized the probe to a 0.6 kbp DNA band, invisible on the ethidium bromide stained gel (**Photograph 8.3.**). The other plasmids did not hybridize this probe. Only pLW1 hybridized a *cpe* specific DNA probe (**Photograph 8.4.**). Even the DNA containing the mixed population of plasmids (Chapter 4), which had hybridized the oligonucleotide probe, did not hybridize this probe. All of the presumptive colonies were subjected to further screening to locate *cpe* clones and to examine the number of clones which hybridized the *Hin*d III fragment. Colonies of *E. coli* cells containing pLW1 or pHG165 served as positive and negative controls. Many of the presumptive positive transformants hybridized the 0.6 kbp *Hin*d III probe (**Photograph 8.2.** Plate C) but non of them hybridized the *cpe* specific probe (**Photograph 8.2.** Plate B).

The *cpe* specific probe, a 520 *Bgl* II/*Sma* I fragment of pLW1, was used to probe transformants containing recombinant pHG165 plasmids with *Pst* I/*Eco* RI inserts. A second copy of the 5' end of *cpe (***Figure 8.2.***)* was located. These transformants were reprobed with a second *cpe* specific probe (**Figure 8.3.** Probe 3) to specifically identify clones containing the 3' *Eco* RI/*Pst* I fragment (**Figure 8.2.**) and to reduce the high background hybridization to vector DNA due to the amount of mcs sequence contained in probe 2. No clones were detected which hybridized this probe.

8. 2. 6. *C. perfringens* DNA Restriction Fragments

Many problems were encountered during the isolation of *C. perfringens* chromosomal DNA (Appendix II). Cell lysis was difficult to achieve, even with the acetone wash and high lysozyme concentrations, and the DNA obtained was frequently unuseable because it was sheared or was resistant to cleavage by restriction enzymes. The resistance to endonuclease was not remedied by phenol:chloroform extractions which succeeded only in shearing the DNA. Reliable supplies of good quality chromosomal DNA were obtained when a NaCl cell prewash with a guanidium thiocyanate DNA extraction method (Appendix II) was introduced

It was thought that the inability to isolate an intact *cpe* clone from the *Hin*d III library and the failure to obtain enough clones with *Eco* RI *C. perfringens* DNA fragments was because some property of the DNA was toxic to *E. coli.* Further attempts to reclone an intact *cpe* were therefore abandoned. DNA sequence elucidation of the cloned fragment of *cpe* (Chapter 5) had revealed a *Pst* I site within the coding region and examination of the possible DNA sequences for the rest of the coding region (**Figure 4.1.**) failed to find any other *Pst* I sites. An attempt was made to clone the rest of *cpe* as a *Pst* I fragment (**Figure 8.2.** Fragment II) using the shotgun approach because, at the time, the size of the *Pst* I

fragments carrying *cpe* were unknown and the 'Failed Ligation' hypothesis (**8. 2. 1. Cloning Strategy**) had not yet been put forward as a possible reason for failure. Insufficient numbers of transformants containing recombinant plasmids were obtained to justify embarking on a colony screening programme.

Renewed attempts to clone an entire enterotoxin gene were made with the adoption of the fragment enrichment strategy. Several clones of seven different recombinant plasmids hybridized the 5.6 kbp pLW1 derived probe but none hybridized a *cpe* specific probe, which again prompted speculation that the expression of *cpe* gene was lethal to *E. coli*. Southern blot analysis of *Pst* I digested DNA with a *cpe* specific probe (**Figure 8.3.** Probe 2) indicated that the 5' end of *cpe* was located on a 12 kbp *Pst* I fragment whilst the required 3' end was on a 9 kbp fragment (**Photograph 8.1.**). Attempts were therefore made to clone *cpe* as a 9 kbp *Pst* I fragment but insufficient numbers of Ap^R Lac^- transformants were obtained. This result, coupled with the observation that three *cpe* cloning strategies [*Iwanejko et al.*, 1989; *van Damme-Jongsten et al.*, 1989 and *Hanna et al.*, 1989] had failed to produce a clone with an intact *cpe*, or with any significant 3' flanking DNA, fuelled speculation that the cytotoxicty may be due to a *cpe* downstream region. One possible explanation for the effect could be expression of a second toxin gene as it was known that the genes for some virulence factors mapped close together on the *C. perfringens* chromosome [*Canard & Cole*, 1989]. The apparent DNA rearrangements observed in the original cloning experiment (Chapter 4), the discovery of a high copy number sequence upstream of the cloned *cpe* (Chapter 9) and the revelation that the genes for the α and θ toxins map close to the attachment site for the lysogenic phage φ29 [*Canard & Cole*, 1989] led to the formulation of the following alternative explanation for the cytotoxic effects of the region. It was postulated that the region contained a mobile genetic element, such as a prophage, able to mediate DNA rearrangements deleterious to the *E. coli* host.

The proposal to clone a shorter fragment, in order to overcome the problems associated with the downstream sequences, was made. It was calculated that the 3' end of *cpe* was located on a 7 kbp *Eco* RI/*Pst* I fragment (**Figure 8.2.** Fragment III). This was verified by Southern blot analysis with a *cpe* probe (**Figure 8.3.** Probe 2) (**Photograph 8.3.**). Chromosomal DNA fragments of 7 kbp ± 2 kbp were cloned into *E. coli* SURE™ . A clone containing the 4.5 kbp *5' Eco* RI/*Pst* I *cpe* fragment was recovered but no clone containing the 3' half of *cpe* was identified, another indication that the DNA 3' to *cpe* was causing the problem.

Other restriction enzymes which would produce an even shorter fragment were then considered. The choice of enzyme was restricted by the availability of suitable sites in appropriate cloning vectors. The range of candidate enzymes was further reduced by the AT rich nature of *C. perfringens* DNA. Most restriction enzymes do not have AT rich recognition sequences, presumably because it is a characteristic common to bacterial promoter sequences and origins of replication. The enzymes with AT rich recognition sequences such as *Hind* III were likely to cleave the chromosomal DNA too frequently. The best candidate enzyme was *Hpa* I with the target site GTT/AAC i.e. would create a blunt end which would be compatible with a *Sma* I created blunt end in the multiple cloning site of pHG165. Further Southern blot analysis of chromosomal DNA (data not shown) identified a *Pst* I/*Hpa* I fragment, ≅ 3 kbp, which could be cloned into the *Pst* I/*Sma* I cleaved multiple cloning site. The recloning programme ended, however due to lack of time.

8. 2. 7. Experimental Controls

The efficient labelling of probe DNA was verified by TCA precipitation (Appendix III) and by acrylamide gel electrophoresis of a small sample of the reaction followed by autoradiography. *E. coli* transformed with pLW1 or pHG165 were the positive and negative hybridization controls included in all colony hybridization assays.

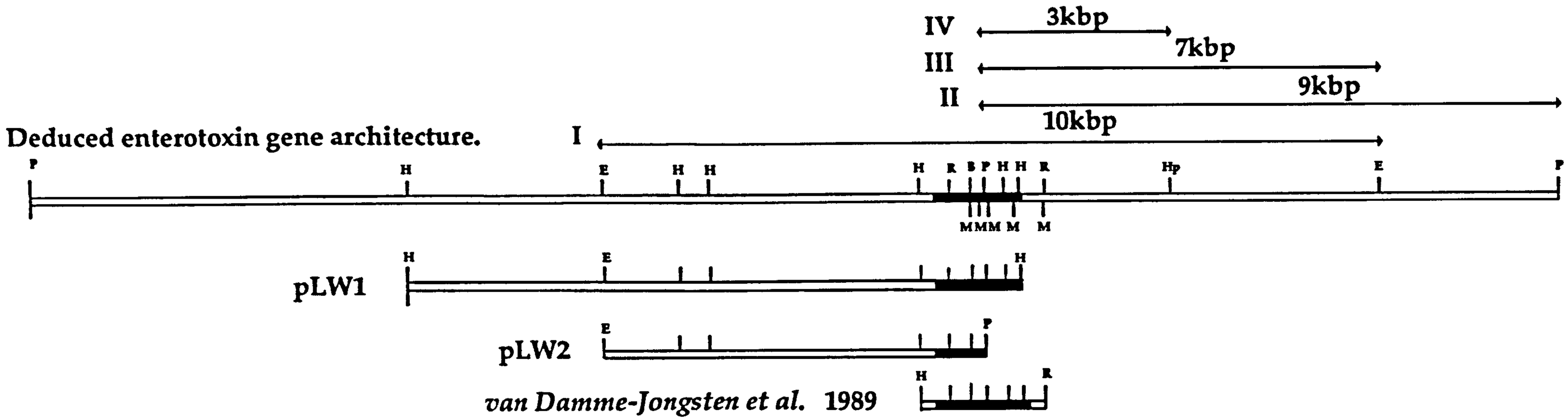
Loss of *C. perfringens* DNA during the fragment isolation and purification procedures was eliminated as the cause of the recloning failures during the final cloning experiments. The *Pst* I/*Hpa* I *cpe* chromosomal DNA fragment was followed during agarose gel isolation and purification by Southern blot analysis of the DNA at the end of each procedure. The DNA was not lost at any stage during the preparation of the fragment DNA.

The quality of linearised vector DNA, the efficacy of the alkaline phosphatase treatment and the efficiency of the ligation reactions were qualitatively assessed by comparing the transformation efficiencies obtained with the following forms of pHG165 DNA:

1. Covalently closed circular.
2. Linearised and agarose gel purified.
3. As 2 and religated.
4. Linearised and alkaline phosphatased to remove the terminal phosphate.
5. As 4 and religated.
6. As 5 and polynucleotide kinased to replace the terminal phosphate.
7. As 6 and religated.

All the plasmid DNAs gave the expected changes in transformation efficiencies apart from the *Pst* I/*Sma* I cut vector. The *Pst* I/*Sma* I cut vector

was not alkaline phosphatase treated because correctly cut DNA would not have the correct ends to religate. It was concluded that *Pst* I and *Sma* I were not cutting pHG165 efficiently when the numbers of transformants obtained from cut DNA were not significantly different to those obtained with the uncut vector. Also agarose gel electrophoresis of vector DNA incubated overnight with T4 DNA ligase did not reveal a band corresponding to the linearised vector but did produce a smeared band characteristic of religated plasmid DNAs. The different optimum reaction conditions of the two enzymes [Pharmacia] and the close proximity of the *Pst* I and *Sma* I sites of pHG165 (Appendix I) may have resulted in the inability to simultaneously cut the polylinker with both enzymes, however various digestion combinations were tried i.e. both enzymes together at two different temperatures; *Pst* I followed by *Sma* I and *vice versa* with and without purifying the DNA between digests; also the concentration of the reaction buffer was varied, without success.

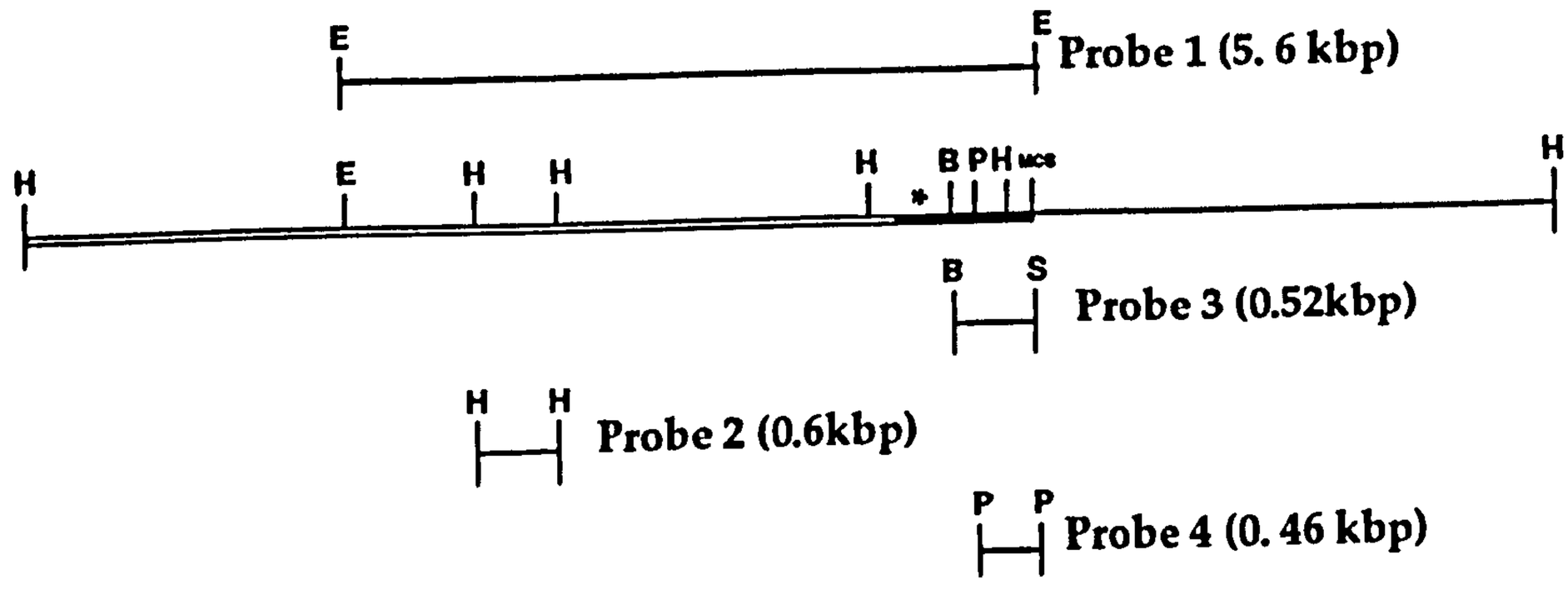


Where H = *Hind* III, E = *Eco* RI, R= *Rsa* I, B = *Bgl* II, M = *Mb0* I, P = *Pst* I, Hp = *Hpa* I

Figure 8. 2.

Architecture of the Enterotoxin Gene, Comparison to the Various *cpe* Clones and Fragments Used in Recloning Strategies

Figure 8. 3.
Restriction Fragments of pLW1 Used as DNA Probe to Screen Transformants for *cpe* Clones



Where *= the position of the oligonucleotide probe used to identify pLW1, H = *Hin*d III, E = *Eco* RI, B = *Bgl* II, P = *Pst* I, S = *Sma* I, MCS = multiple cloning site.

Photograph 8. 2.
Colony Hybridization of *E. coli* Transformants with the 5.6 kbp *Eco* RI Fragment of pLW1.

Plate A

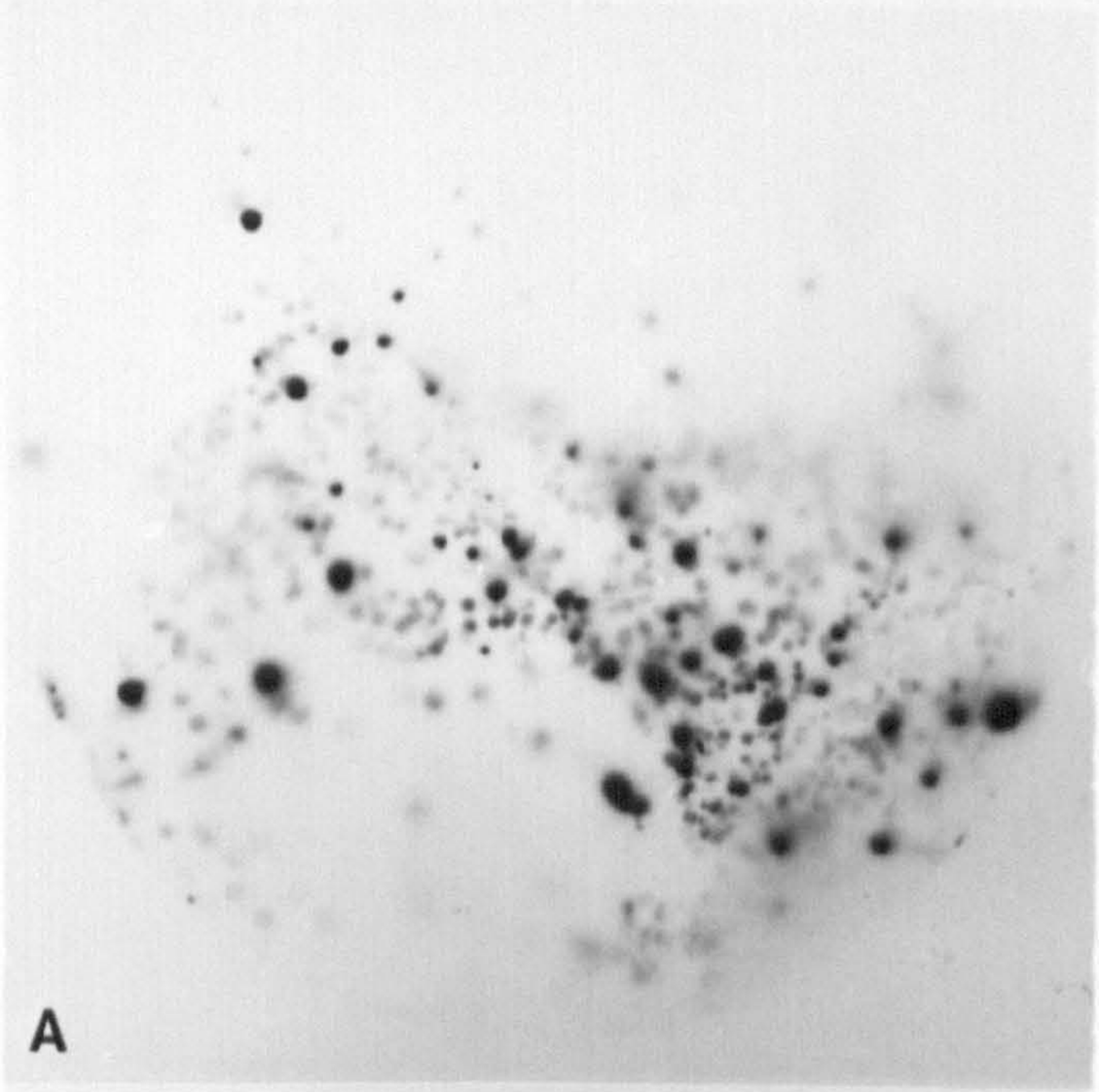
One of five plates of *E. coli* SURE™ transformed with pHG165 containing ≅10 kbp inserts of *Eco* RI digested *C. perfringens* chromosomal DNA. The colonies were hybridized with a 5.6 kbp fragment of pLW1 containing the cloned portion of *cpe*.

Plate B

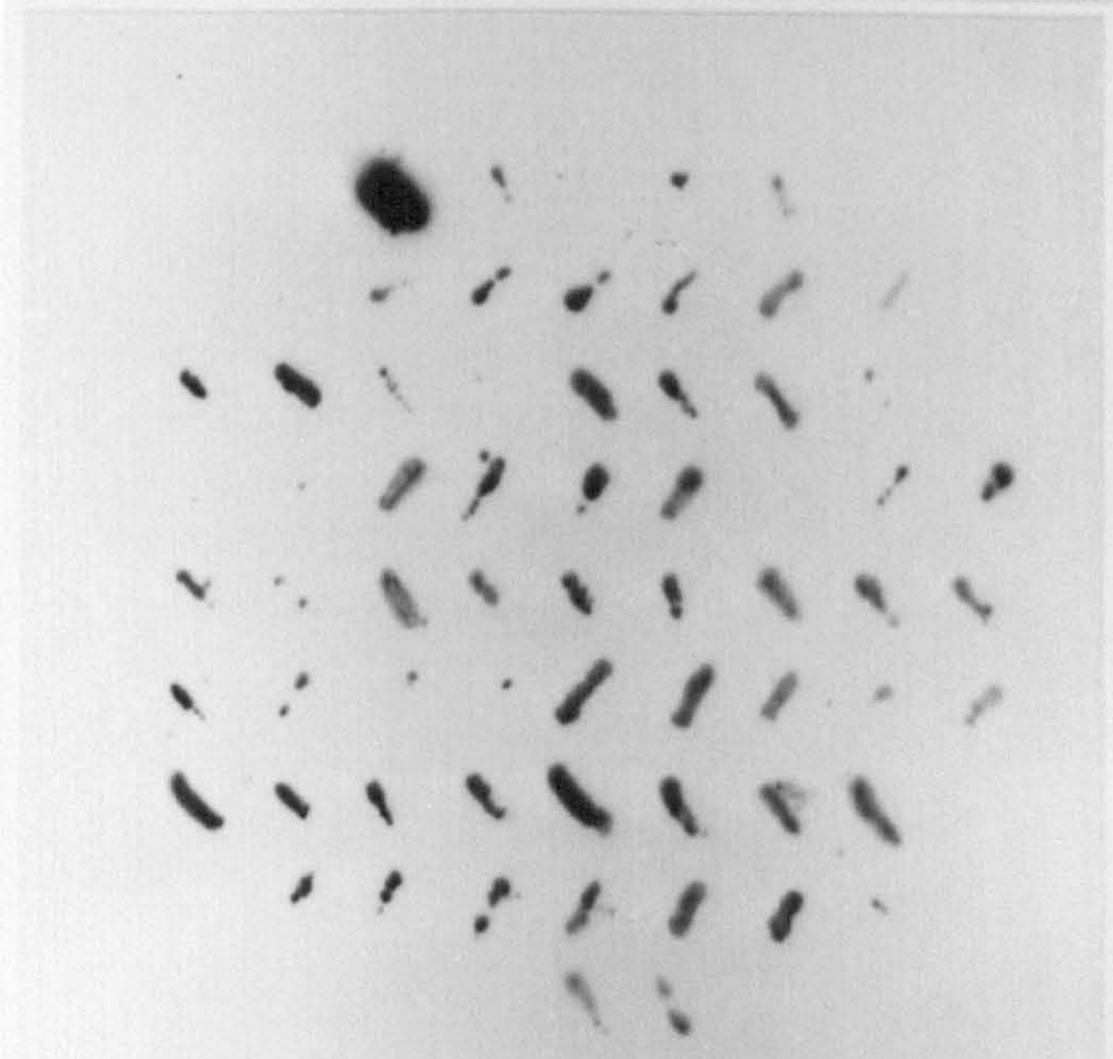
One of the plates of *cpe* presumptive positive clones hybridized with a *cpe* specific probe. pLW1, the *cpe* positive control was the only colony that hybridized the probe (Top left).

Plate C

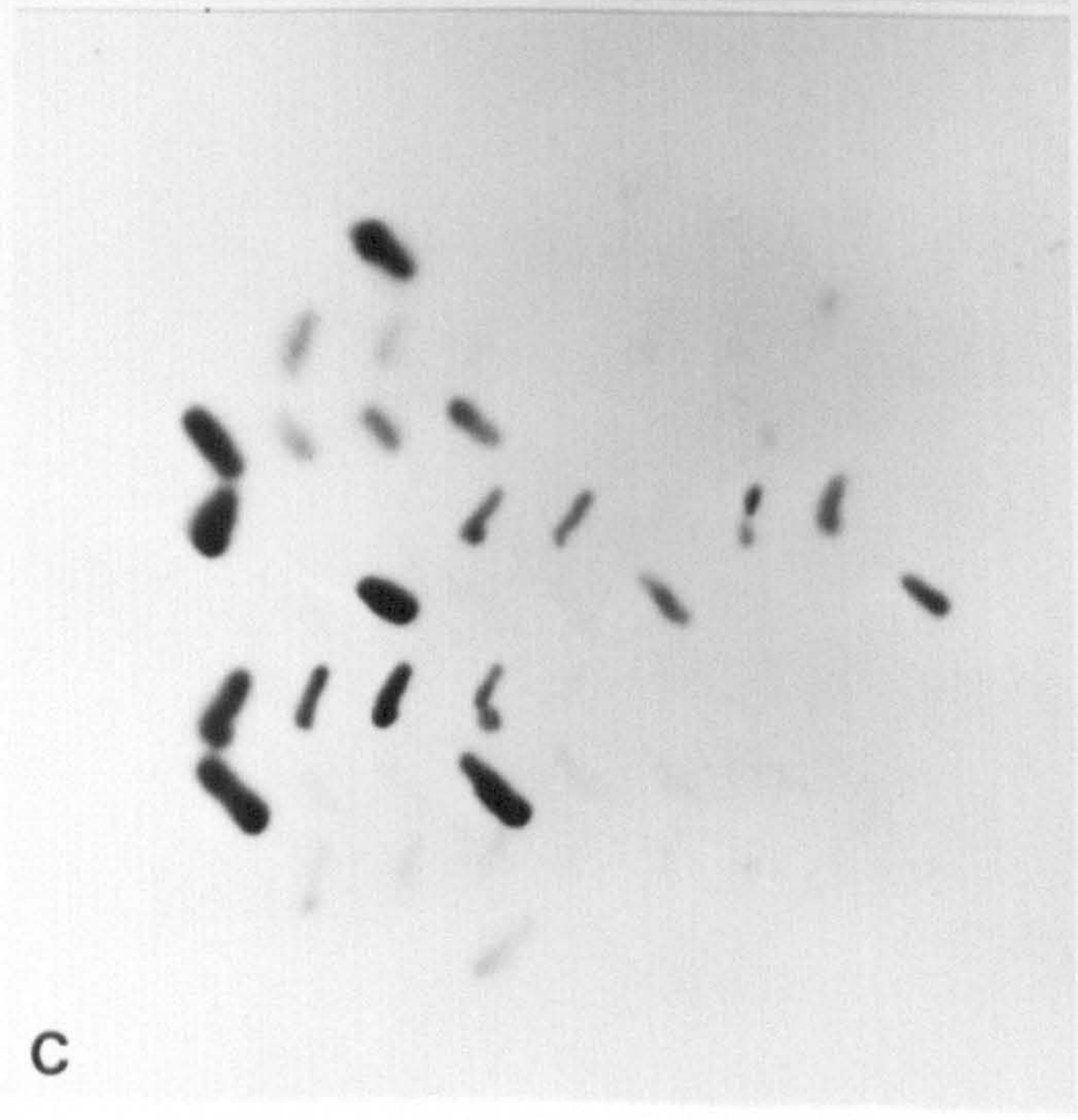
Colonies on plate C were replicas of those on plate B. The colonies were hybridized with a 0.6 kbp *H*ind III fragment located upstream of the cloned *cpe* gene on pLW1. Several colonies, including pLW1, hybridised the probe.



A



B

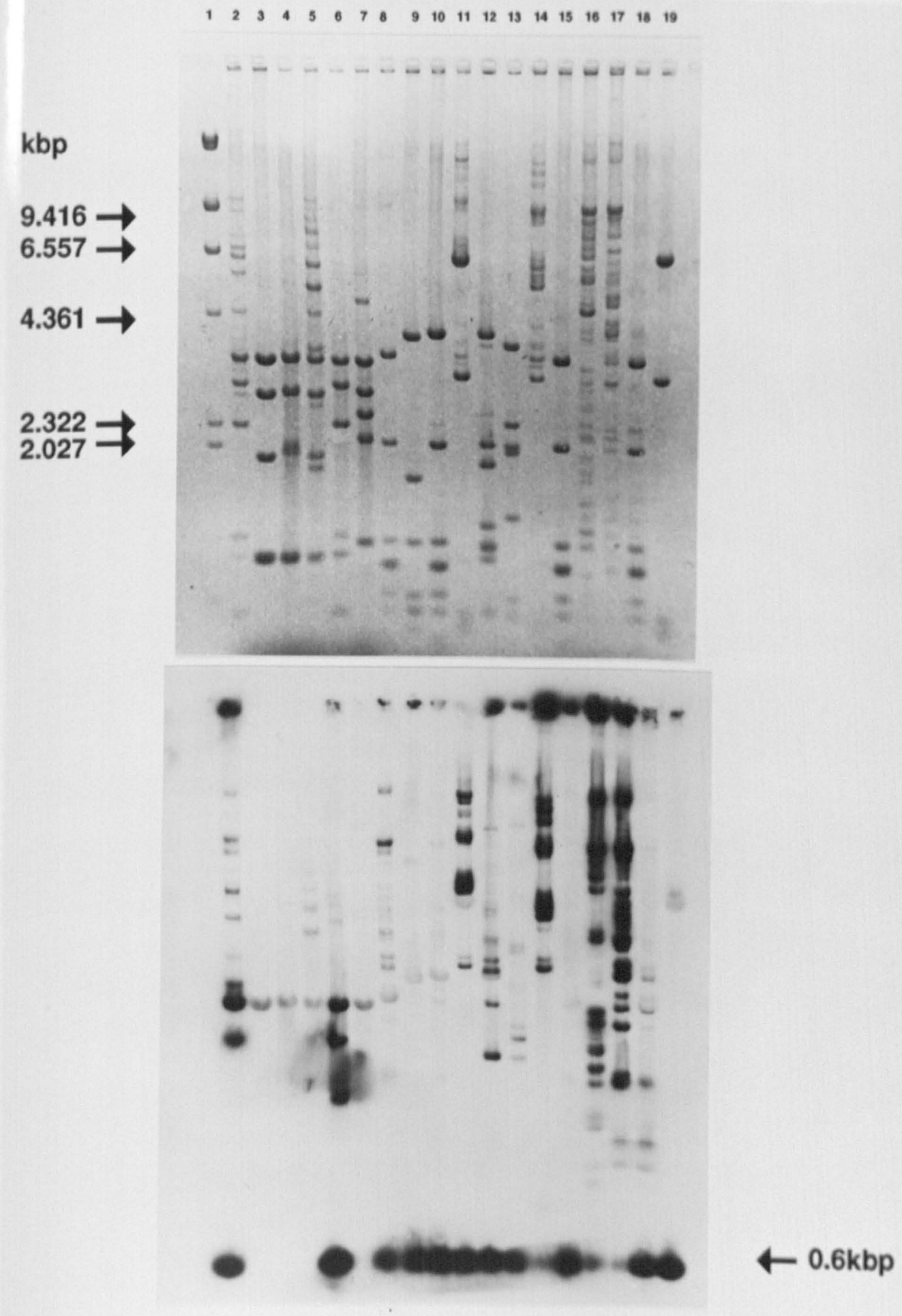


C

Photograph 8. 3.
A *Hin*d III Digest of Plasmid DNA from a Random Selection of Presumptive *cpe* Positive Clones

Lane	Description
1	*Hin*d III digested bacteriophage λ DNA
2	*Hin*d III digested pLW1 DNA
3,4,5,6	*Hin*d III digested plasmid DNA obtained from stabs of the original clone types (Chapter 4).
7-19	*Hin*d III digests of plasmid DNA from a random selection of presumptive *cpe* positive clones.

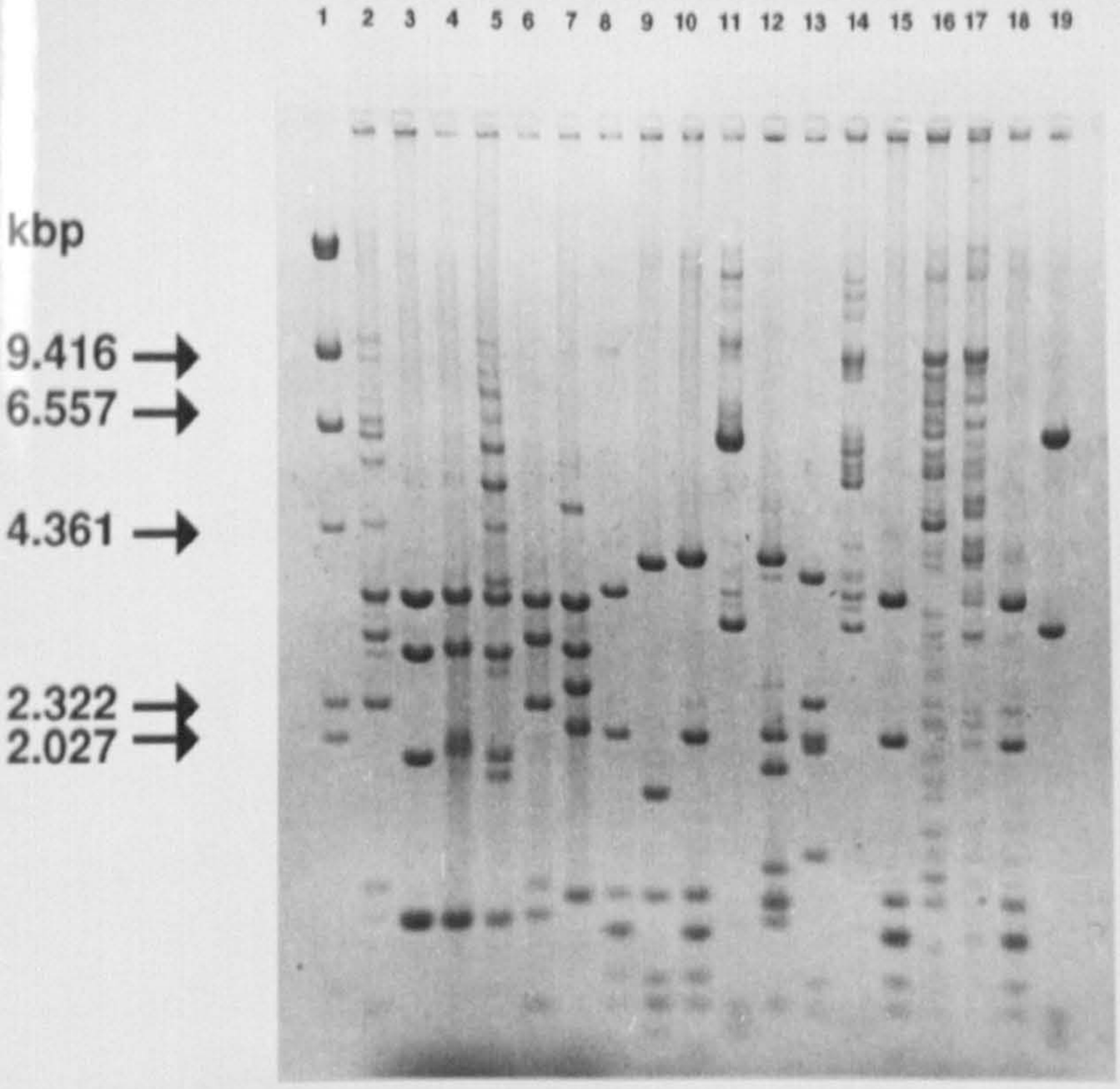
Plasmids were separated by agarose gel electrophoresis (top) and blotted onto nylon membrane. The filter was hybridized with an 0.6 kbp *Hin*d III fragment of pLW1 and autoradiographed (bottom).

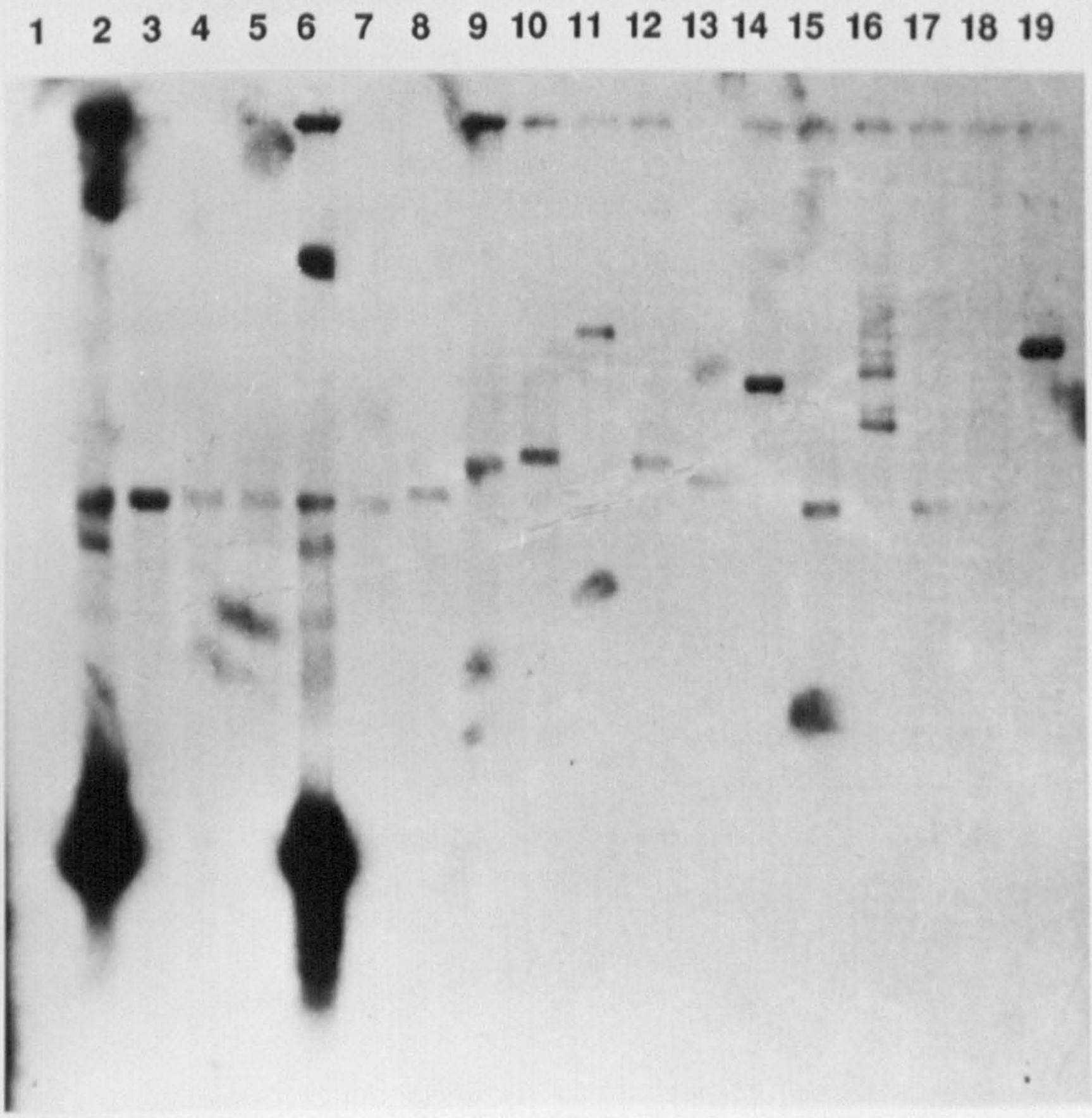

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
kbp
9.416
6.557
4.361
2.322
2.027
0.6kbp

Photograph 8. 4.
A *Hin*d III Digest of Plasmid DNA from a Random Selection of Presumptive *cpe* Positive Clones

Lane	Description
1	*Hin*d III digested bacteriophage λ DNA
2	*Hin*d III digested pLW1 DNA
3,4,5,6	*Hin*d III digested plasmid DNA obtained from stabs of the original clone types (Chapter 4).
7-19	*Hin*d III digests of plasmid DNA from a random selection of presumptive *cpe* positive clones.

Plasmids were separated by agarose gel electrophoresis (top) and blotted onto nylon membrane. The filter was hybridized with a *cpe* specific probe and autoradiographed (bottom).


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
kbp
9.416
6.557
4.361
2.322
2.027


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
890bp

8. 3. Discussion

Evidence discounting experimental error as the reason for the inability to isolate a clone of the 3' end of *cpe* was provided by the recloning of the 5' end of *cpe* and the cloning of several independent *Eco* RI fragments of the *C. perfringens* NCTC 8239 chromosome (Chapter 9). Also the observation that three other cloning experiments [Chapter 4; *van Damme-Jongsten et al.*, 1989; *Hanna et al.*, 1989] failed to produce a *cpe* clone with a sizeable amount of downstream DNA is an indication that the DNA may be 'unclonable' using contemporary cloning methods. Although the cloning strategy published by van Damme-Jongsten *et al.* would not have produced a clone with a significant amount of 3' flanking DNA because it involved complete digestion of the chromosome with enzymes that, perhaps fortuitously, cleaved the DNA just downstream of *cpe*. The other strategies however would have been expected to produce such a clone.

Possible biological explanations for the 'unclonability' of *cpe* associated DNA are based on current knowledge of *C. perfringens* biology, the reactions of DNA and gene expression in *E. coli*.

The DNA could code for a protein which is toxic to *E. coli*. *C. perfringens* produce a number of cytotoxins (Chapter 2) the genes for which could be located close to *cpe* [*Canard & Cole*, 1989]. Also *E. coli* and *C. perfringens* cohabit in the human gastrointestinal tract so it is not infeasible to postulate that they are competitors and as such may produce bacteriocins against one another. Uncontrolled expression of genes which do not code for normally toxic proteins e.g. genes for regulatory proteins, could also be deleterious.

Insert mediated DNA rearrangements could be lethal. Intermolecular recombination, such as transposition events, resulting in integration of insert sequences into the host chromosome would probably cause a lethal disruption of normal gene expression. Intramolecular illegitimate recombination would cause deletion or inversion of vector sequences which may prevent its maintenance in the cell. Illegitimate recombination has been associated with DNA replication events which involve the production of ssDNA e.g. bacteriophage M13 [*Michel & Ehrlich*, 1986] and repeated DNA sequences. Evidence in favour of this explanation came from the unexpected observation that the mixed plasmid population from one of the original clones that had previously contained a band complementary to a short oligonucleotide probe homologous to the 5' end of *cpe* (Chapter 4) did not hybridize the DNA fragment for the 3' end of the cloned *cpe*. This DNA did not hybridize the 0.6 kbp *Hin*d III probe either.

The base composition of the insert could also affect the cell. It would have a

global affect on gene expression if, for instance, it had a high affinity for DNA binding proteins. If the insert carried powerful promoters or if it altered the topology of the plasmid DNA then it would possibly detrimentally affect vector DNA sequences i.e. structural genes, origins of replication and regulatory regions.

It is impossible to known which, if any, of these explains the inability to clone *cpe* downstream flanking DNA into *E. coli*. An answer may only be obtained when it becomes possible to reliably clone into *C. perfringens* or other closely related organism.

CHAPTER 9

IDENTIFICATION OF A NOVEL DNA FRAGMENT PRESENT IN HIGH COPY NUMBER ON THE CHROMOSOMES OF SOME *C. PERFRINGENS* STRAINS

9.1. Introduction

An attempt to clone the enterotoxin gene on a ≅10 kbp *Eco* RI fragment (Chapter 8) resulted in the production of a significant number of transformants that hybridized the 5.6 kbp DNA probe (**Figure 9.1.**). None of them hybridized the 520 bp *Bgl* II/*Sma* I *cpe* specific probe but they all hybridized a 0.6 kbp *Hin*d III fragment located ≅ 3 kbp 5' to the cloned enterotoxin genes on pLW1 and pLW2.

A *Hin*d III digestion of the recombinant plasmids contained within twelve of the clones identified several different plasmids. The subsequent Southern blot analysis revealed the presence of the 0.6 kbp *Hin*d III fragment in all the plasmids examined.

This result could have arisen as a cloning artifact i.e. *in vitro* ligation of fragments that are not contiguous *in vivo* but there are two reasons why this was unlikely to happen. Firstly, only fragments in the 9-10 kbp range were isolated for cloning, a double insertion would have produced a construct too large for successful transformation. Secondly, non of the recombinant plasmids contained more than one *Eco* RI insert which was as expected since the fragments were produced from completely restricted DNA. The various restriction patterns could have arisen by *in vivo* DNA rearrangement of a parent plasmid giving several daughter plasmids, such reactions of the DNA would have to have been mediated by the insert because the host cells, *E. coli* SURE™ (Appendix I) were Rec⁻. If it was a real phenomenon then the *Hin*d III fragment would be located on several chromosomal *Eco* RI fragments equivalent in size to the inserts.

A complex hybridization pattern would emerge if the fragment was part of a dispersed gene family such as the rRNA operons [*Canard & Cole*, 1989]. It would also be expected that all strains of *C. perfringens* would contain homologous rDNA and would also give complex hybridization patterns with the *Hin*d III fragment as the probe.

9. 2. Methods

9. 2. 1. A Complex Hybridization Pattern

Chromosomal DNA was extracted from *C. perfringens* NCTC 8239 using the GES method (Appendix II). One sample was digested with *Hin*d III whilst a second sample was restricted with *Eco* RI. The DNAs were separated by agarose gel electrophoresis **(Photograph 9.1. Lanes 3 & 6.)** *Hin*d III digested bacteriophage λ and pLW1 DNAs as size markers and controls **(Photograph 9.1. Lanes 1 & 2.)**. The DNA was blotted onto Hybond N+ as described previously (Chapter 7. Experiment 2). The filter was then probed with the ^{32}P labelled *Bgl* II/*Sma* I fragment of *cpe* **(Figure 9.1.)**, to ensure that the DNA was not partially digested, using the DNA labelling and hybridization protocols described earlier (Chapter 7. Experiment 2).

After autoradiography **(Photograph 9.1. Lane 4.)** the filter was stripped of bound probe by submerged it in boiling 0.1% SDS and leaving to cool to room temperature. Successful stripping was confirmed by autoradiography. The filter was then reprobed using the 0.6 kbp *Hin*d III fragment **(Figure 9.1.)**. Procedures for probe labelling, hybridization and autoradiography **(Photograph 9.1. Lanes 5 & 7.)** were as before.

9. 2. 2. Comparison of Insert Size to Chromosomal DNA Bands

A selection of the clones which had hybridized the 5.6 kbp *Eco* RI DNA probe, during the attempt to clone *cpe* as ≅10 kbp*Eco* RI fragment (Chapter 8), were chosen for further analysis. Plasmid DNA was extracted using the small scale alkaline lysis method (Appendix II). A sample of each plasmid and *C. perfringens* NCTC 8239 chromosomal DNA was digested with *Eco* RI and electrophoresed on an 0.8% agarose gel with the usual size markers and hybridization control DNAs **(Photograph 9.2. (A))**. A second plasmid and chromosomal DNA sample was similarly digested with *Hin*d III and electrophoresed **(Photograph 9.3. (A))**. The two gels were blotted onto Hybond N+ and probed with the 460 bp *Pst* I enterotoxin gene probe **(Figure 7.2.)** as described previously (Chapter 7. Experiment 2), and autoradiographed **(Photographs 9.2. (B) & 9.3. (B))**.

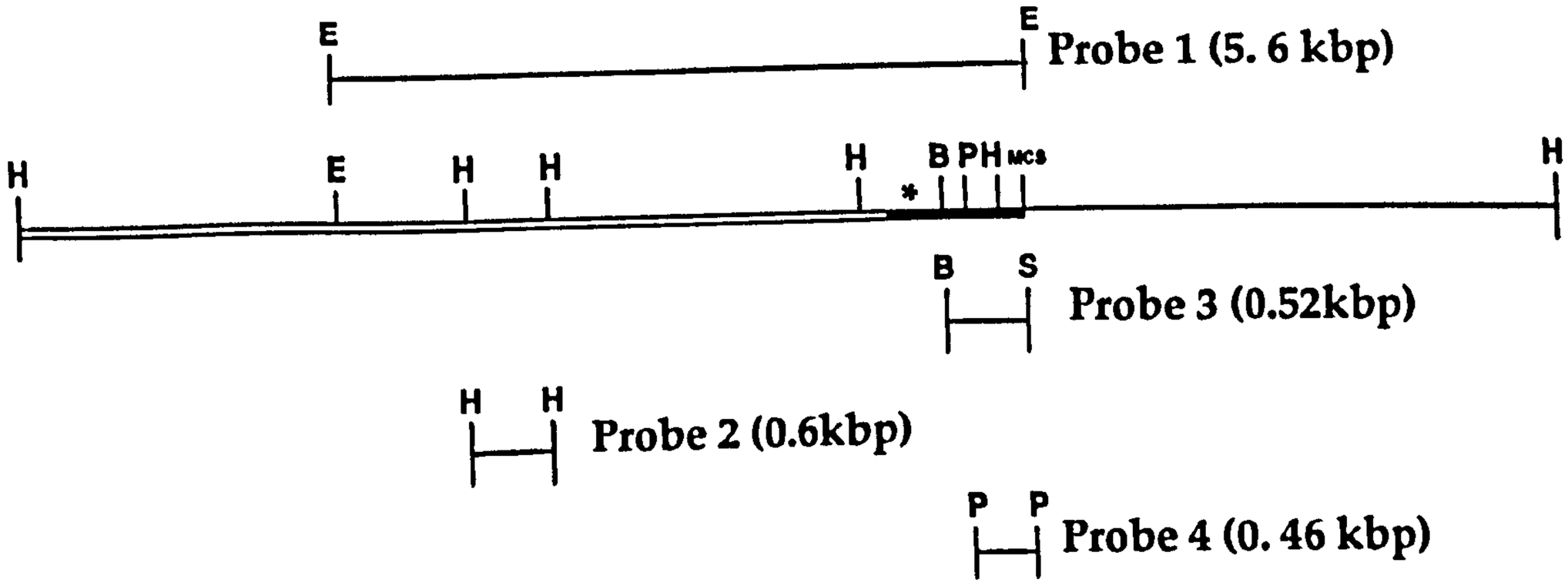
The filters were then stripped of bound probe (this chapter. Experiment One), reprobed with the 0.6 kbp *Hin*d III fragment and a second autoradiograph obtained **(Photographs 9.2. (C) & 9.3. (C))**.

9. 2. 3. Interstrain Comparison

Previously prepared filters of *C. perfringens* DNAs (Chapter 7. Experiment 3.) **(Photographs 9.4. & 9.5.)** were stripped, as described earlier, of bound enterotoxin gene probe and reprobed with the 0.6 kbp *Hin*d III **(Figure 9.1.)** and autoradiographed **(Photographs 9.4. & 9.5.)**

Figure 9. 1.

Location of the Novel DNA Fragment with Respect to the Cloned Enterotoxin Gene pLW1

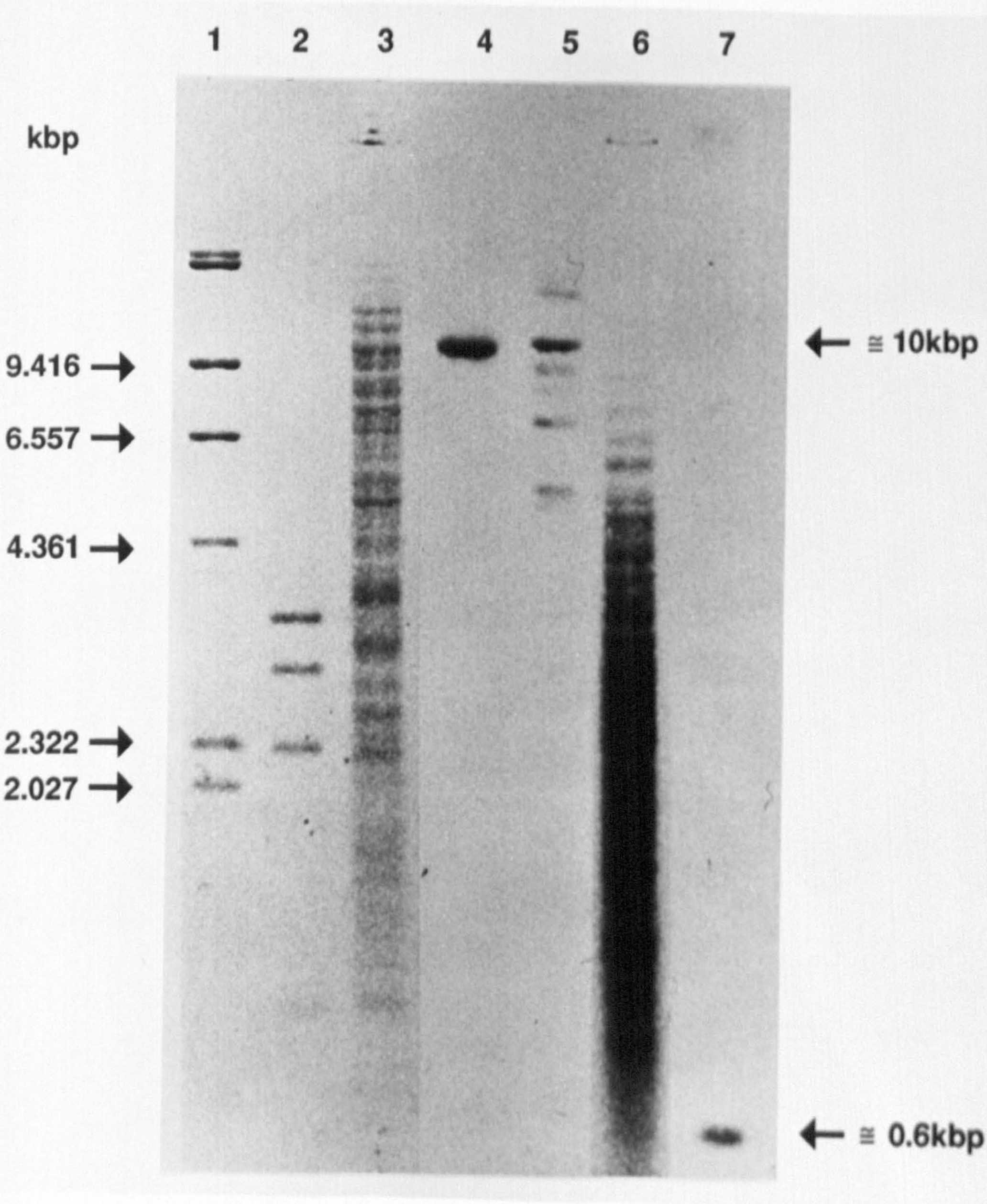


Where H = *Hin*d III, E = *Eco* RI, B = *Bgl* II, P = *Pst* I, S = *Sma* I, mcs = multiple cloning site.

Photograph 9. 1.

Identification of Multiple Copies of a Novel DNA Fragment and a Single Copy of the Enterotoxin Gene on the Chromosome of the Enterotoxin Positive *C. perfringens* Type A Strain NCTC 8239

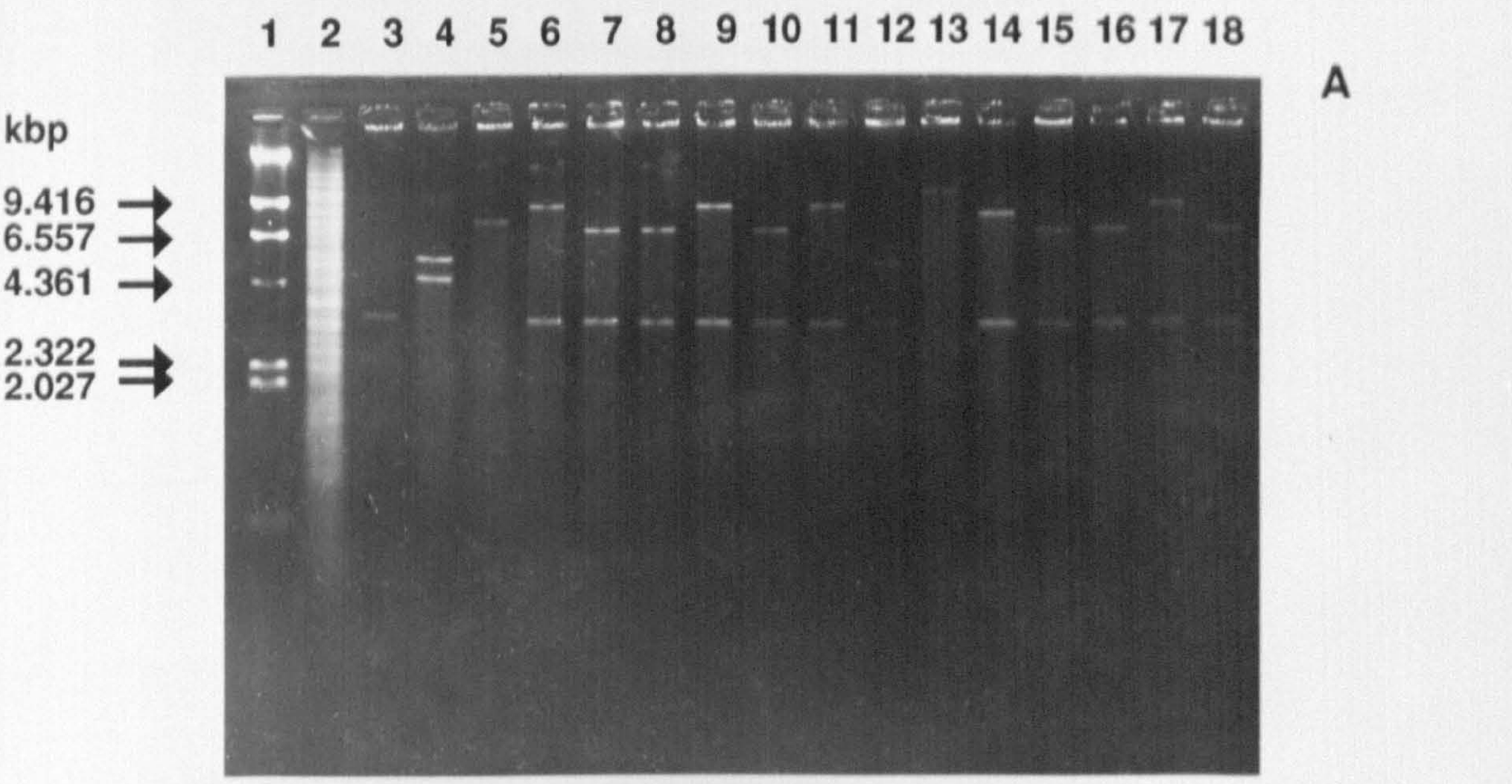
Lane	Description
1	Agarose-ethidium bromide gel of *Hin*d III digested bacteriophage λ DNA
2	Agarose-ethidium bromide gel of *Hin*d III digested pLW1 DNA
3	Agarose-ethidium bromide gel of *Eco* RI *C. perfringens* NCTC 8239 DNA
4	A Southern blot of lane 3 probed with the *Bgl* II / *Sma* I Fragment
5	A Southern blot of lane 3 probed with the *Hin*d III Fragment
6	Agarose-ethidium bromide gel of *Hin*d III *C. perfringens* NCTC 8239 DNA
7	A Southern blot of lane 6 probed with the *Hin*d III Fragment

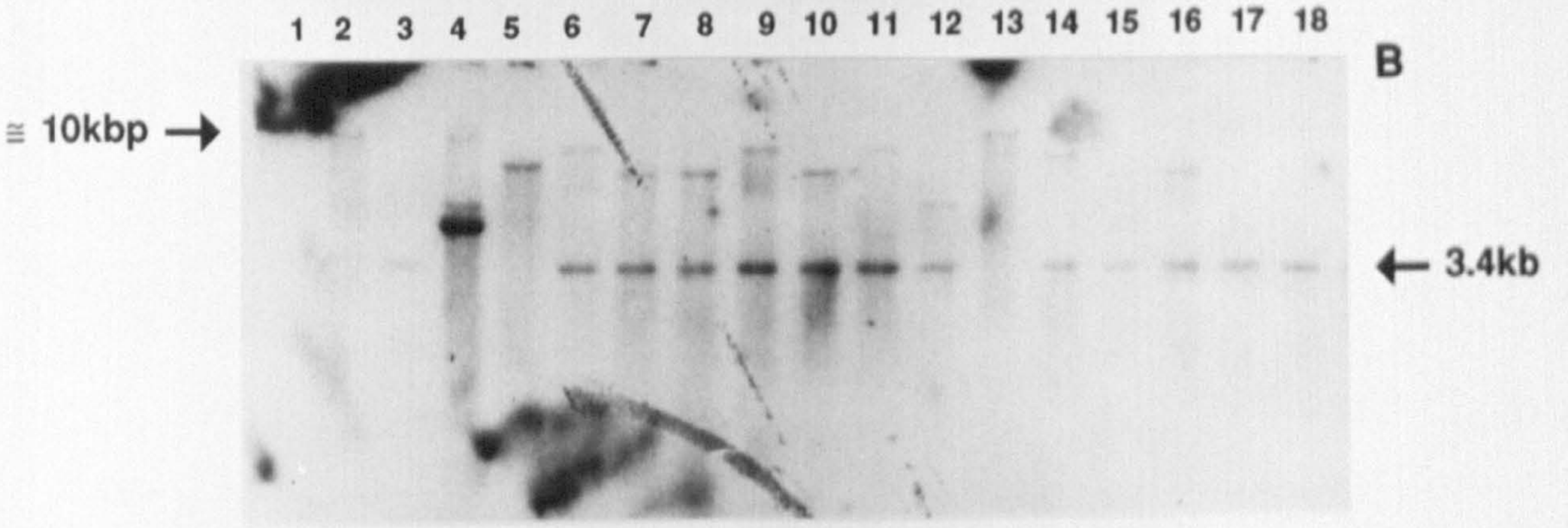

1
2
3
4
5
6
7
kbp
9.416
6.557
4.361
2.322
2.027
≅ 10kbp
≅ 0.6kbp

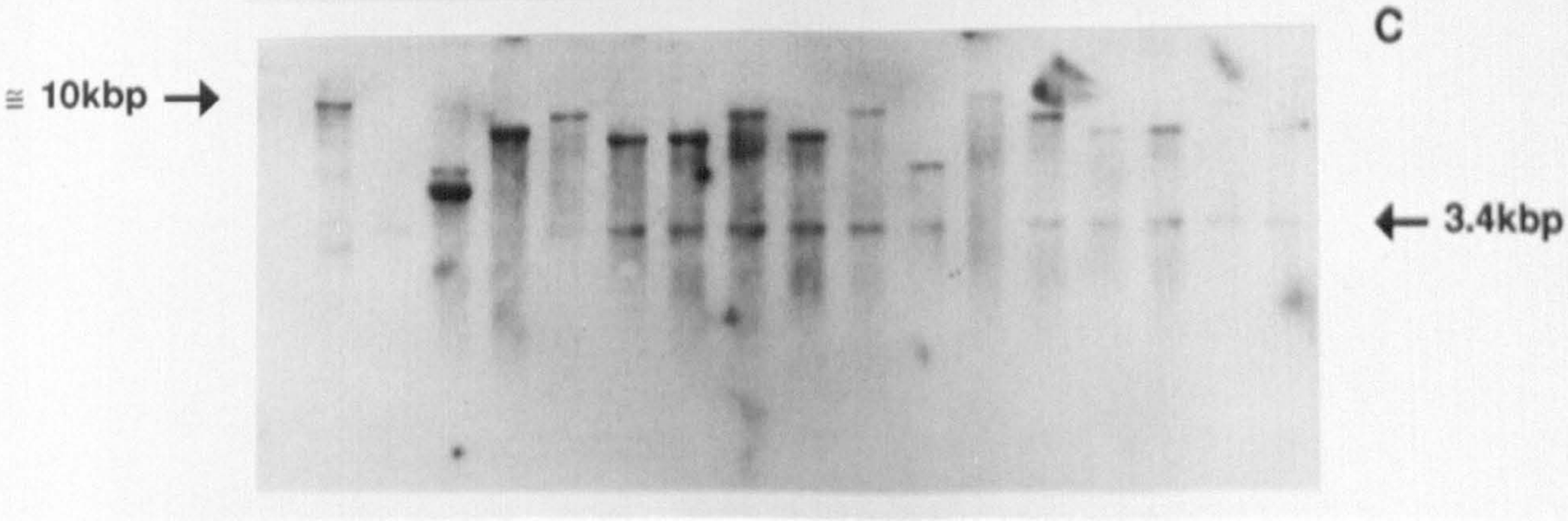
Photograph 9. 2.
Eco RI Digested Plasmid and Chromosomal DNAs (A) Probed with the *Pst* I Enterotoxin Gene Probe (B) and the Novel *Hin*d III Fragment (C)

Lane	Discription
1	*Hin*d III bacteriophage λ DNA
2	*Eco* RI digested *C. perfringens* NCTC 8239 chromosomal DNA
3	*Hin*d III digested pLW1
4	*Eco* RI digested pLW1

The remaining tracks contain *Eco* RI digested recombinant plasmids.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
A
kbp
9.416
6.557
4.361
2.322
2.027

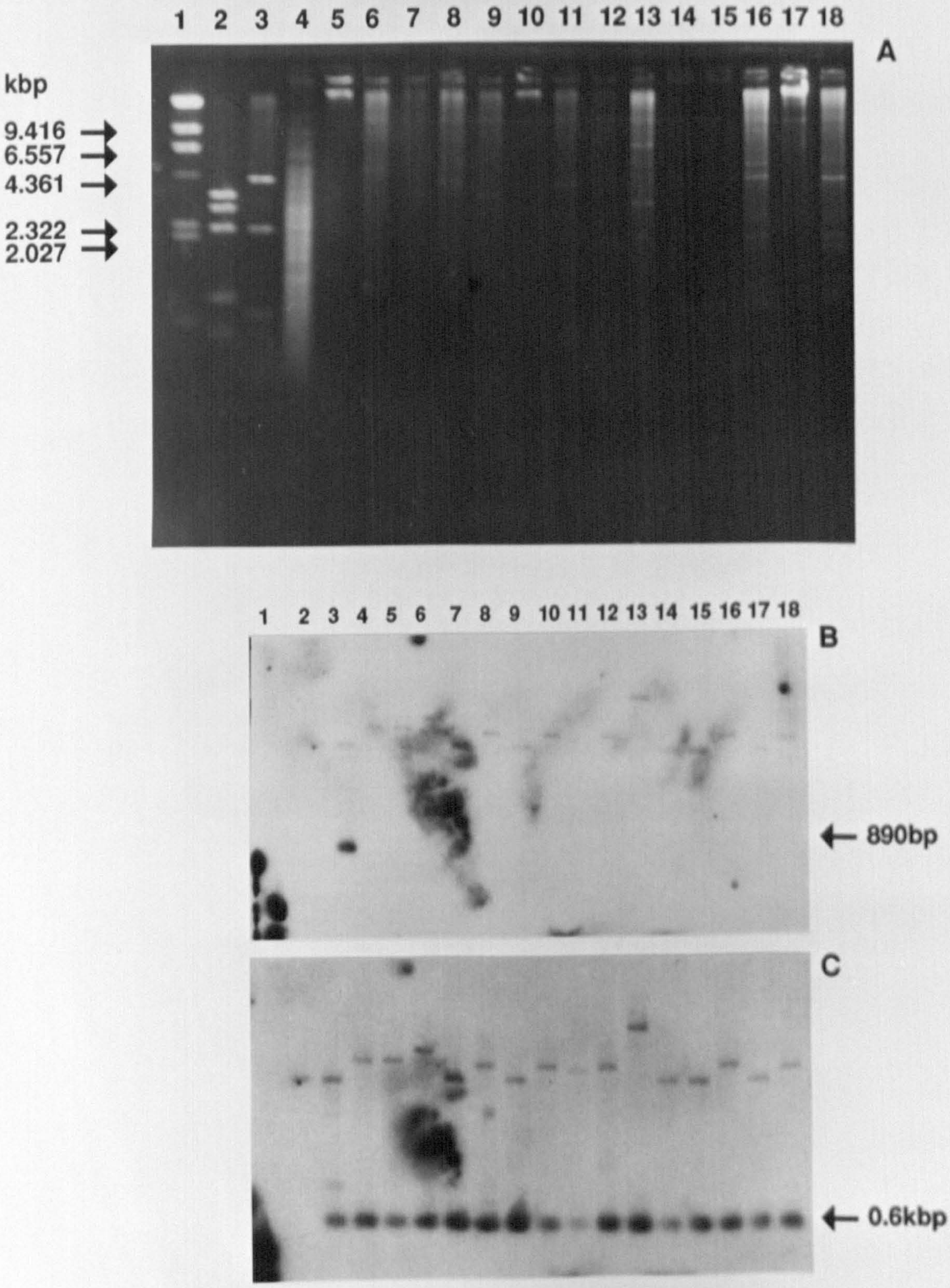

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
≅ 10kbp
3.4kb

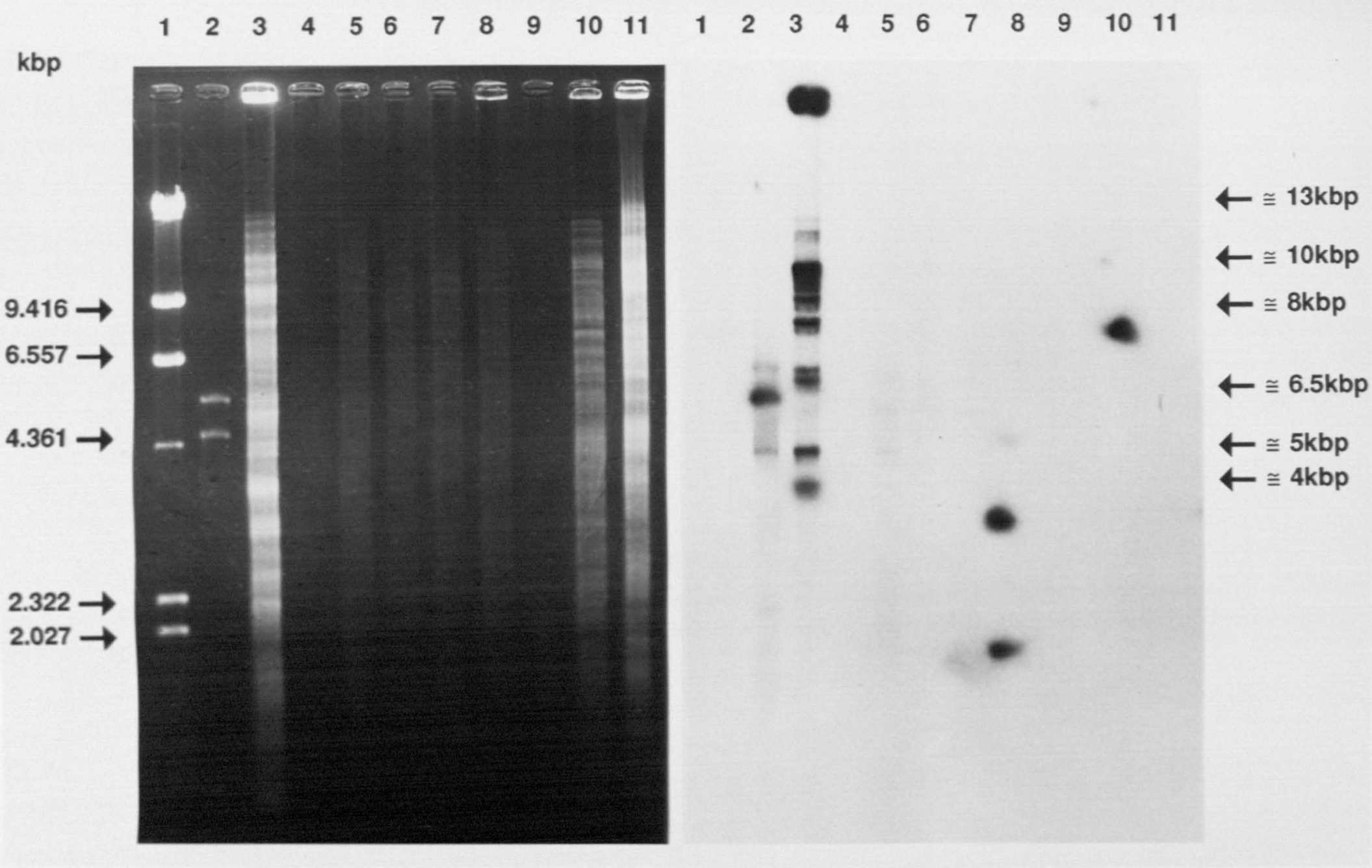

C
≅ 10kbp
3.4kbp

Photograph 9. 3.
*Hin*d III Digested Plasmid and Chromosomal DNAs (A) Probed with the *Pst* I Enterotoxin Gene Probe (B) and the Novel *Hin*d III Fragment (C)

Lane	Discription
1	*Hin*d III bacteriophage λ DNA
2	*Hin*d III digested pLW1
3	*Hin*d III digested pLW2
4	*Hin*d III digested *C. perfringens* NCTC 8239 chromosomal DNA

The remaining tracks contain *Hin*d III digested recombinant plasmid


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
A
kbp
9.416
6.557
4.361
2.322
2.027
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
← 890bp
C
← 0.6kbp


1 2 3 4 5 6 7 8 9 10 11
kbp
9.416
6.557
4.361
2.322
2.027
1 2 3 4 5 6 7 8 9 10 11
≅ 13kbp
≅ 10kbp
≅ 8kbp
≅ 6.5kbp
≅ 5kbp
≅ 4kbp

Photograph 9. 4.

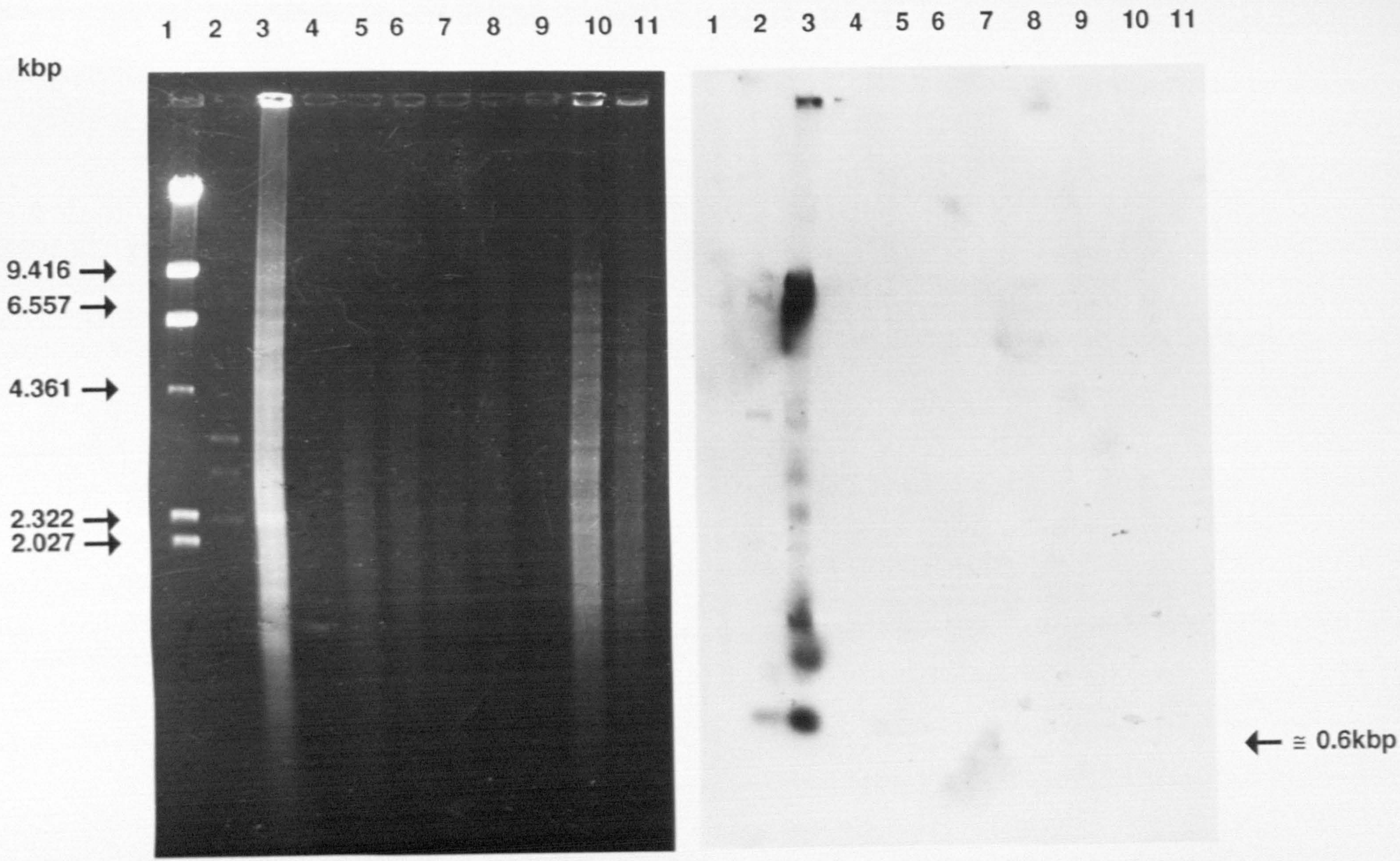
Probing the *Eco* RI Digested DNA from Different *C. perfringens* Isolates and Toxin Types for the Novel DNA Fragment

Lane	Description of the DNA
1	*Hind* III digested bacteriophage λ DNA
2	*Eco* RI digested pLW1
3	*Eco* RI *C. perfringens* NCTC 8239 (has no detectable plasmids)
4	*Eco* RI *C. perfringens* NCTC 8449 (has no detectable plasmids)
5	*Eco* RI *C. perfringens* NCTC 10239 **(has one plasmid ≅ 6.0 kbp)**
6	*Eco* RI *C. perfringens* NCTC 10613 (has no detectable plasmids)
7	*Eco* RI *C. perfringens* type A strain 2498 (plasmid content unknown)
8	*Eco* RI *C. perfringens* type C strain 5381 (plasmid content unknown)
9	*Eco* RI *C. perfringens* type C strain 5383 (plasmid content unknown)
10	*Eco* RI *C. perfringens* type D strain 755 (plasmid content unknown)
11	*Eco* RI *C. perfringens* F1415 **(has one plasmid ≅ 6.4 kbp)**

Photograph 9. 5.

Probing the *Hin*d III Digested DNA from Different *C. perfringens* Isolates and Toxin Types for the Novel DNA Fragment

Lane	Description of the DNA
1	*Hin*d III digested bacteriophage λ DNA
2	*Hin*d III digested pLW1
3	*Hin*d III *C. perfringens* NCTC 8239 (has no detectable plasmids)
4	*Hin*d III *C. perfringens* NCTC 8449 (has no detectable plasmids)
5	*Hin*d III *C. perfringens* NCTC 10239 **(has one plasmid ≅ 6.0 kbp)**
6	*Hin*d III *C. perfringens* NCTC 10613 (has no detectable plasmids)
7	*Hin*d III *C. perfringens* type A strain 2498 (plasmid content unknown)
8	*Hin*d III *C. perfringens* type C strain 5381 (plasmid content unknown)
9	*Hin*d III *C. perfringens* type C strain 5383 (plasmid content unknown)
10	*Hin*d III *C. perfringens* type D strain 755 (plasmid content unknown)
11	*Hin*d III *C. perfringens* F1415 **(has one plasmid ≅ 6.4 kbp)**


1 2 3 4 5 6 7 8 9 10 11
kbp
9.416
6.557
4.361
2.322
2.027
1 2 3 4 5 6 7 8 9 10 11
≅ 0.6kbp

9. 3. Results and Discussion

The 0.6 kbp *Hin*d III probe gave a complex hybridization pattern with *C. perfringens* NCTC 8239 *Eco* RI cut DNA (**Photograph 9.1.**). This was not due to an incomplete digestion of the chromosomal DNA because only one band hybridized the *cpe* probe. The many copies of the DNA fragments which contained the *Hin*d III fragment were assumed to be identical since a complex pattern was not observed with *Hin*d III digested chromosomal DNA.

Southern blot analyses confirmed the ubiquitous presence of the 0.6 kbp fragment amongst the recombinant plasmids and the complete absence of a cloned enterotoxin gene (**Photographs 9.2. & 9.3.**). Chromosomal *Eco* RI fragments equivalent in size to the inserts hybridized the 0.6 kbp *Hin*d III probe, evidence that the cloned fragments truly reflected chromosomal DNA fragments.

Chromosomal DNA from several toxin type A strains gave complex but differing hybridization patterns with the *Hin*d III probe (**Photograph 9.4.**) and a single, similarly sized *Hin*d III band (**Photograph 9.5.**). The toxin types C and D did not hybridize the probe nor did toxin type A strains NCTC 8449 and F1415, disputing the idea that the fragment forms part of a conserved rDNA gene family.

A mobile genetic element explanation for the observations has been proposed and is discussed in the next chapter, along with the possible effects such an element could have on *cpe* expression. It is unlikely that the element is the previously described Tn*4451* or Tn*4452* [*Abraham & Rood*, 1987] because they do not contain,what in this case appears to be a highly conserved, 0.6 kbp *Hin*d III fragment.

DISCUSSION
CHAPTER 10

10. 1. Introduction

The *C. perfringens* interstrain and intrastrain variation in enterotoxin production is consistent with the involvement of a putative mobile genetic element in *cpe* expression. There is still insufficient data available to fully support such a relationship but the results from observations presented in this thesis show some striking similarities with known systems. Precedents have been set linking mobile elements with clostridial virulence factors. Namely the correlation between neurotoxin synthesis and an 81 MDa plasmid in strains of *C. botulinum* type G [*Ecklund et al.*, 1988], the bacteriophage interconversion of *C. botulinum* types C and D [*Ecklund & Poysky*, 1974] and bacteriophage influenced toxin production by *C. novyi* types A and B [*Ecklund et al.*, 1976].

10. 2. Salient Features of Mobile Genetic Elements

Bacteria may contain, or acquire, autonomously replicating, expendable, extrachromosomal genetic elements such as, plasmids,. transposons, bacteriophages and insertion sequences (IS) [*Reanney*, 1976; *Cambell*, 1981]. These elements are able to alter the phenotype of the host by effecting genotypic changes resulting in altered gene expression. The alterations may be brought about by genome reorganization, insertional gene inactivation or polarity effects of integrated elements on distal genes [*Berg & Howe*, 1989]. Gene expression is affected by DNA rearrangements that affect gene dosage, such as duplications and deletions [*Anderson & Roth*, 1977; *Schmid & Roth*, 1987] and events such as translocation [*Schmid & Roth*, 1987], inversion [*Glasgow et al.*, 1989] and integration/excision e.g. the *xyl* genes on the TOL plasmid, pWW0, of *Pseudomonas putida* MW1000 [*Sinclair et al.*, 1986] that result in gene positioning effects.

The processes via which extrachromosomal elements may effect genomic rearrangements are:- host mediated homologous recombination between repeated sequences such as IS elements [*Galas & Chandler*, 1989]; site-specific, 'resolvase' mediated, homologous recombination such as bacteriophage λ integration and excision [*Thompson & Landy*, 1989] and excision of Tn*5* and Tn*10* [*Egner & Berg*, 1981; *Foster et al.*, 1981; *Berg et al.*, 1983]; 'transposase' mediated replicative transposition e.g. phage Mu [*Pato*, 1989] and conservative transposition [*Berg*, 1983] and 'transposase' mediated one-ended transposition of Tn*3* and phage Mu [*Ehrlich*, 1989].

Potentially lethal DNA rearrangements may be caused by unregulated transposition events. In consequence there are a variety of regulatory mechanisms that regulate the mobility of transposable elements, minimising the danger to the host yet maximising the survival and distribution of the element. Bacteriophage λ for example employs a sophisticated system, that encompasses most levels of control, and enables the lysis/lysogeny switch to be determined by both the environment and host cell physiology [*Thompson & Landy*, 1989]. Transcriptional control of transposition genes by a protein repressor auto-regulates transposition of the highly mutagenic bacteriophage Mu [*Pato*, 1989] and the Tn*3* subfamily of transposons. The Tn*3* repressor, the product of the *tnpR* gene, also doubles as the resolvase. It is able to regulate its own synthesis because the *tnpR* promoter lies within the resolvase DNA binding site I of the *res* region [*Sherrat*, 1989]. Transposition of Tn*5*, Tn*9* and Tn*10* is limited by the preferential *cis* action of the transposase which prevents mobilization of distant copies of the element [*Isberg & Syvanen*, 1981]. Tn*10* (IS*10*) transposition is translationally regulated by a *trans* acting antisense RNA which binds to a complementary sequence on the transposase mRNA preventing ribosome binding and encouraging digestion by RNase III [*Sasakawa*, 1982; *Kleckner*, 1989]. Post-translational regulation of Tn*5* transposition is effected by a *trans*-acting protein inhibitor of the transposase activity [*Yin & Reznikoff*, 1987]. Two regulatory mechanisms at the DNA level link transposition events with DNA replication. Firstly, the IS*10* and IS*50* transposases are sensitive to DNA methylation, preferring unmethylated DNA, able to utilise hemi-methylated DNA as a substrate but not able to use fully methylated DNA [*Yin et al.*, 1988; *Roberts et al.*, 1985; *Dodson & Berg*, 1989]. Secondly, DnaA protein is needed for both Tn*5* (IS*50*) mobilization and initiation of bacterial chromosome replication [*Phadnis & Berg*, 1987; *Yin & Reznikoff*, 1987]. The majority of mechanisms limit mobilization to newly occupied or distressed cells.

The classification of bacterial transposable elements into IS elements, composite transposons, Tn*3* like elements and bacteriophages becomes almost arbitrary when the similarities that exist between most of the elements and the lack of certain distinguishing features in others is considered. The genetic organization of phage Mu for instance can be described as modular [*Cambell & Botstein*, 1983] and overall it has similarities with that of bacteriophage λ. The invertible G region shares extensive homologies with the C region of phage P1 and the flagellar phase variation system in *Salmonella* spp. and all three invertases Gin, Cin and Hin, are very similar to the Tn*3* resolvase [*Simon*, 1980]. The phage Mu transposition mechanism is also closely related

to that of Tn*3* [*Pato*, 1989]. Many other elements encode resolvases which appear to have a common evolutionary origin, it comes as no surprise therefore to find that their sites of action, *res* regions, are also very similar [*Sherrat*, 1989]. The architecture of the equivalent site in the *C. perfringens* plasmid, pIP404, (termed the RES region) is similar to the Tn*3* *res* region but there are no detectable DNA sequence homologies [*Garnier et al.*, 1987]. A phylogenetic tree derived from the primary structures of recombinases and resolvases has shown that the pIP404 resolvase is most closely related to the resolvase of the class II, Tn*3*-like, transposon, Tn*917*, of *Streptococcus faecalis* [*Shaw & Clewell*, 1985]. Other 'novel' elements detected in Gram-positive organisms lack terminal inverted repeats and variable termini and they do not generate duplications of the target sequences on insertion, yet they still share extensive homologies with Gram-negative elements. Tn*554* of *Staphylococcus aureus*, for example, is a site-specific transposon that has homologies with temperate bacteriophage, whilst Tn*916* (*Enterococcus* [*Streptococcus*] *faecalis*) and Tn*1545* (*Streptococcus pneumoniae*) encode their own transference systems and therefore have homology with Gram-negative conjugative plasmids [*Murphy*, 1989].

10. 3. Comparison of Extrachromosomal Genetic Elements Characteristics with those of *cpe*.

Detailed below are both the observations and the arguments that support an association between an extrachromosomal element and the *C. perfringens* enterotoxin gene, *cpe*.

I. Extrachromosomal elements by definition are expendable and may only be maintained if they confer a selective advantage to the host such as the penicillin resistance conferred on clinical isolates of *S. aureus* by the plasmid encoded *blaZ* gene [*Shalita & Novick*, 1980]. The expendability of *cpe* was demonstrated by Southern blot analyses [This publication; *Phillips Jones et al.*, 1989] and colony blot hybridization studies [*van Damme-Jongsten et al.*, 1989 and 1990] which failed to detect *cpe* in the majority of the *C. perfringens* strains examined.

II. Transposition of some elements is closely associated with stressed and/or damaged cells e.g. bacteriophage λ [*Thompson & Landy*, 1989]. CPE appears to have close links with stressed and/or damaged cells. CPE synthesis is associated with sporulation, a physiological response to stress. Also the correlation between food poisoning incidents, which are mostly attributable to the consumption of cooked food (Chapter 2), and possession of *cpe* links

CPE with cells subjected to heat damaging treatment.

III. The Shapiro model for intermolecular replicative transposition of Tn*3* [*Sherrat*, 1989], bacteriophage Mu during lytic growth [*Pato*, 1989] and sometimes IS*1* [*Galas & Chandler*, 1989; *Biel & Berg*, 1984] proposes the introduction of single-strand DNA nicks at the 3' ends of the elements and staggered nicks in the target DNA producing 5' overhangs. The free 3' ends of the donor molecule are ligated to the 5' ends of the recipient DNA molecule producing a branched structure similar to a replication fork. The free 3' ends of the target DNA then prime DNA replication of the element. The resulting intermediate cointegrate structure is then resolved (see below) in to the products of the transposition event. Aberrant, one ended transposition has been recorded for Tn*3*-like elements [*Sherrat*, 1989]. The single available 3' end of the element ligates to the target DNA as before, but because there is only one primer of DNA replication, rolling circle DNA replication ensues and the normal cointegrate cannot be formed. Eventually an abberant second cut is introduced in to the newly replicated donor DNA molecule which is followed by ligation to the target DNA.

Plasmids define their own copy number in bacterial cells by regulating their own replication. As both replicons employ the same replisome their rates of replication must be the same therefore the difference in copy number of the various plasmid types (incompatibility groups) and the chromosome must be due to differences in the frequencies of initiation of DNA synthesis. The frequency of pHG165 replication is regulated by a 555 nucleotide RNA primer of DNA synthesis (RNA II), the availability of which is controlled by the combined actions of an antisense RNA molecule (RNA I) and a protein repressor (ROP). RNA I and ROP therefore control pHG165 copy number (Appendix I) by regulating the frequency of initiation of plasmid DNA replication at the Col E1 *ori*. They would not affect DNA replication initiated at other origins e.g. the rolling circle replication described above, a possible explanation for the apparent alteration in copy number of the pHG165 derivative, pLW1 (Chapter 4).

IV. Replicative transposition involves cointegrate formation and resolution. Resolution results from a resolvase mediated site-specific recombination event between two directly repeated *res* regions (inverted repeats results in inversion of the intervening sequence e.g. Gin, Hin and Cin.). As discussed earlier, considerable homologies exist between the different *res* sites of a variety of elements, including the RES site of the *C. perfringens* UV inducible, bacteriocinogenic plasmid, pIP404. The recombination mechanisms are assumed to be the same for all cases [*Sherrat*, 1989]. A resolvase dimer is

presumed to bind to each of several bipartite resolvase binding sites present in the region. The DNA binding of individual monomers is facilitated by the C-terminal domain, which has a classic helix-turn-helix motif. The N-terminal domains are presumed to be responsible for protein-protein interactions and DNA strand exchange. The use of several resolvase binding sites is thought to be necessary to bring into juxtaposition distant crossover points. DNA sequencing of the cloned *cpe* has identified two direct repeats of sequences homologous to the cross over points of the Tn*3* subfamily of transposons (Chapter 5) [*Sherrat*, 1989]. The DNA region separating the two sites contained two interesting sequences, one was homologous to the pIP404 RES site II left arm, the other was homologous to the right binding arm of site IV. Although the separation and context of the two arms is very different to that observed for pIP404, it may be that the binding and interaction of proteins at these sites could bring the two 'crossover' sequences into position, especially given the probable bent conformation of the intervening DNA (Chapter 5). Recombination between these two sites would have removed the region of the CPE gene used to derive the 26 bp oligonucleotide probe for *cpe* (**Figure 10.1**). Insert mediated recombination of the transforming plasmids, in the *recA*⁻ hosts used in the original cloning experiments, may explain why only one of the plasmids isolated from the original clone hybridized the probe. It is perhaps an indication that the insert carries a resolvase gene. The resolvase binding sites were in similar positions to the two sequences that have homology with the putative -35 sequences of the tetanus toxin gene (Chapter 5) and this probably indicates that the homology is superficial and due to the protein binding capacities of the two sites. The plasmid context of the sites is, therefore, likely to have influenced their behaviour so conclusions regarding the mobility of *cpe in vivo* cannot be reliably drawn from this observation. There are two further lines of support for an insert encoded resolvase. Firstly, the observation that most clones did not contain multiple plasmids may be a reflection of a resolvase based autoregulated system of transposition control. Secondly, the problem did not present itself when different restriction enzymes (*Eco* RI and *Pst* I) were used to reclone the same end of *cpe*, perhaps an indication that the resolvase gene was inactive due to cleavage by the enzyme (see below). The possibility however that the altered genotype of the host cells in the later experiments prevented host mediated DNA rearrangements cannot be ruled out.

V. Mobile genetic elements are typically associated with multiple chromosomal copies e.g. phage Mu [*Pato*, 1989]. A 0.6 kbp *Hind* III fragment located 5' to both copies of the cloned *cpe* was found to have a high copy

number in several strains of *C. perfringens* examined by Southern blot analyses (Chapter 9). The apparent absence of the sequence from some of the strains discounted the idea that the fragment was part of the *C. perfringens rrn* locus. *rrn* loci are often highly repeated but the nucleotide sequences of rRNAs are highly conserved enabling their use as a molecular clock [*Woese*, 1987] (Chapter 1). Referring back to the previous section, restriction mapping of pLW1 (the larger of the two clones) shows that the restriction enzyme *Eco* RI used to clone pLW2 (the smaller *cpe* clone) would have cleaved the DNA ≅ 1 kbp 5' to the 0.6 kbp fragment and could therefore easily have cut within the putative transposable element.

10. 4. Conclusion

There is growing evidence that the expression and interstrain distribution of *cpe* is linked to the activities of a mobile genetic element. There are several other situations were a mobile genetic element has been implicated in the expression of exoproteins. The enterotoxin (*entB*) and TSST-1 genes of *S. aureus* are both thought to be carried on transposable elements because *entB* can be transferred to *rec*⁻ strains, TSST-1 maps to several chromosomal locations and both are generally absent from non producing strains i.e. only maintained when needed [*Shafer & Iandolo*, 1980; *Kreiswirth et al.*, 1985]. More recently a possible IS element has been located upstream of the exotoxin A gene of *Pseudomonas aeruginosa* [*Pritchard & Vasil*, 1990]. Like CPE, exotoxin is not produced by all strains and those that do synthesis it, do so to a greater or lesser extent. The observed hyper-toxin production by some strains could be the result of IS polar effects on gene expression.

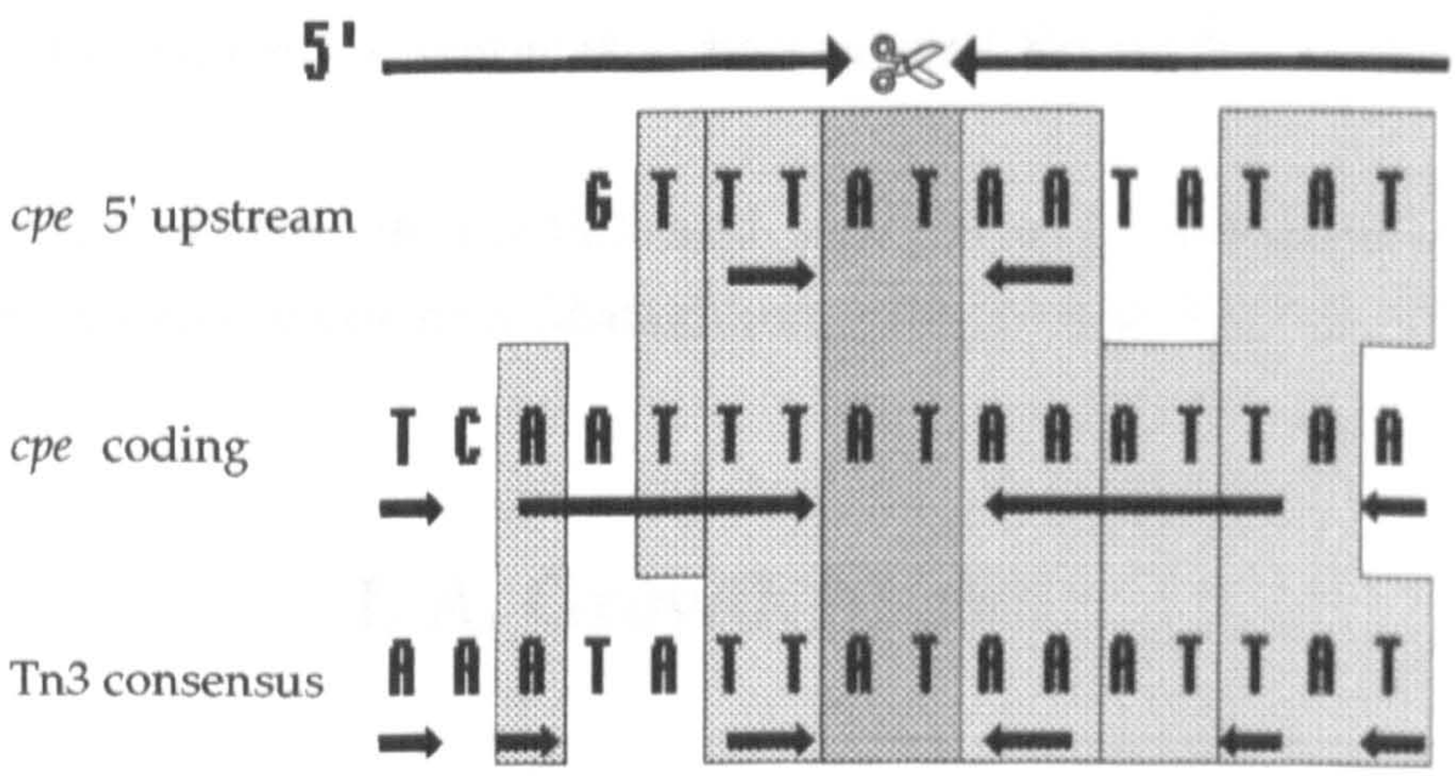
The failure to detect a plasmid borne copy of *cpe*, the single gene copy number in *cpe*⁺ strains and the close vicinity of a high copy number element are indications that mobilization of *cpe* is mediated by a plasmid free conjugative gene transfer mechanism e.g. the transfer of antibiotic resistance mediated by the conjugal streptococcal transposons Tn*916* and Tn*1545* [*Murphy*, 1989]. The only known transposable elements of *C. perfringens* Tn*4451* and Tn*4452* [*Abraham & Rood*, 1987 and 1988] are both associated with conjugal gene transfer. The observed interstrain size conservation of the 0.6 kbp *Hin*d III fragment and the lack of such a fragment from either Tn*4451* or Tn*4452* counts against their involvement in *cpe* expression.

Confirmation of the mobile genetic element hypothesis for *cpe* expression may be obtained by the mapping of the *C. perfringens* genome. Two types of linkage maps would be necessary. A physical map i.e. Southern blot analyses of large chromosomal DNA fragments (separated by pulsed field gel electrophoresis) would map *cpe* to a particular restriction fragment or

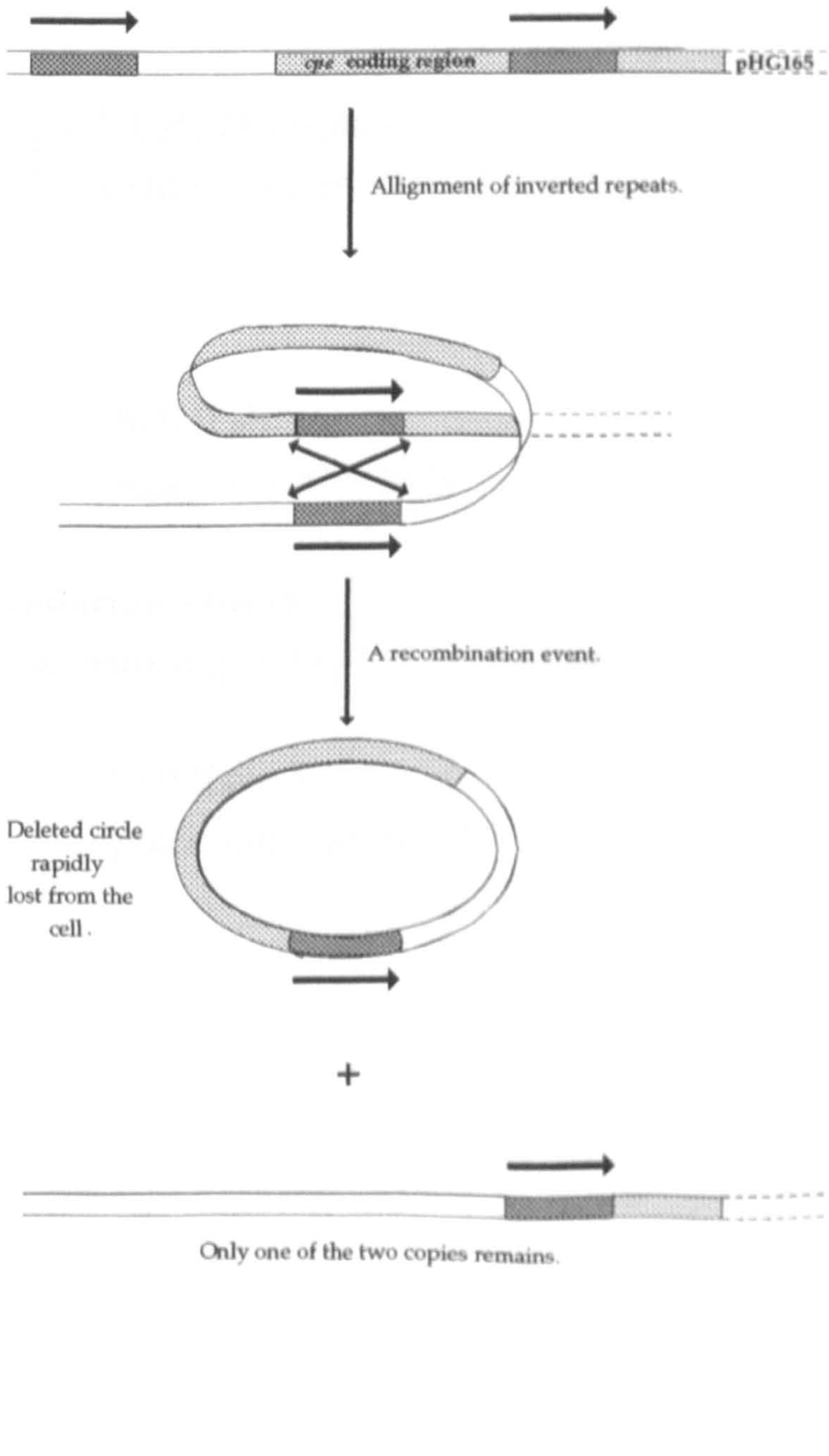
fragments [*Canard & Cole*, 1989]. A classical genetic map i.e linkage analysis using mutant and conjugative *C. perfringens* strains, would determine what phenotypes are linked with the CPE+ phenotype. Physical mapping would detect the integration and excision of a mobile element next to *cpe* because they would cause variation in the flanking DNA. Movement of *cpe* would be detected by both mapping techniques as it would map to several locations.

Figure 10. 1.

Comparison of Two Short Sequences from the Coding and Upstream Regions of *cpe* with the Recombination Site of the Tn3 *res* Region



Possible Result of Recombination between those Two Sites



APPENDIX I
MATERIALS

All medias, agars, buffers and other solutions were made up to the required volume with RO water i.e. water that had passed through a reverse osmosis unit .

Unless otherwise stated all medias and buffers were sterilised either in a Prestige™ pressure cooker or a Matachana autoclave at 121 ° C, 15 psi for 20 minutes.

I. A. Growth Media

Brain Heart Infusion (BHI) Broth.

BHI [Lab. M]	37.0 g l^{-1}

BHI formula;
BHI solids 17.5 g l^{-1}, Lab. M tryptose 10.0 g l^{-1}, dextrose 2.0 g l^{-1}, disodium phosphate 2.5 g l^{-1}, sodium chloride 2.0 g l^{-1}, pH 7.4 .

BHI Agar.

BHI [Lab. M]	37.0 g l^{-1}
Agar N° 1 [OXOID]	15.0 g l^{-1}

BHIN (Nitrate reduction) Broth.
Used in the nitrate reducing ability test of presumptive *C. perfringens* isolates.

BHI [Lab. M]	37.0 g l^{-1}
potassium nitrate [FISONS]	0.1 %

Lactose-Gelatin (LG) Medium.

Used to confirm the lactose fermenting and gelatin hydrolysing abilities of presumptive *C. perfringens* isolates.

tryptose [Lab. M]	15.0 g l^{-1}
yeast extract [DIFCO]	10.0 g l^{-1}
(dibasic) sodium phosphate [FISONS]	5.0 g l^{-1}
phenol red	0.05 g l^{-1}
Agar Nº 1 [OXOID]	12.0 g l^{-1}
gelatin [DIFCO]	12.0 g l^{-1}

Luria-Bartini (LB) Broth.

A general *E. coli* gowth medium.

tryptone [DIFCO]	10.0 g l^{-1}
yeast extract [DIFCO]	5.0 g l^{-1}
sodium chloride [FISONS]	5.0 g l^{-1}
1M sodium hydroxide [FISONS]	4.0 ml
(optional glucose	1.0 g l^{-1})

L Plates.

LB broth	1000.0 ml
agar [DIFCO]	15.0 g l^{-1}

MacConkey Agar.

A media which contains bile salts to inhibit the growth of non-enteric microorganisms. A pH indicator dye, bromocreosol purple, which changes colour (yellow) in an acidic environment, is used to screen for lactose fermenting colonies.

MacConkey broth (purple)[OXOID]	40.0 g l^{-1}
Agar Nº1 [OXOID]	15.0 g l^{-1}

MacConkey broth formula;

peptone 20.0 g l^{-1}, lactose 10.0 g l^{-1}, bile salts 5.0 g l^{-1}, , bromocreosol purple 0.001 g l^{-1},sodium chloride 5.0 g l^{-1}, pH 7.4 ± 0.2 .

Antibiotics.

Added to LB broth, L agar and MacConkey agar, after sterilisation, to select for antibiotic resistant colonies.

Antibiotic	**Final concentration**
ampicillin [SIGMA]	30.0 mg l^{-1}

(stock solution made in sterile RO water)

tetracycline [SIGMA]	10.0 µg ml^{-1}

(stock solution made up in 70% ethanol)

Reinforced Clostridial Medium (RCM).

RCM [OXOID]	38.0 g l^{-1}

RCM formula; yeast extract 3.0 g l^{-1}, Lab-lemco powder 10.0 g l^{-1}, peptone 10.0 g l^{-1}, soluble starch 1.0 g l^{-1}, dextrose 5.0 g l^{-1}, cysteine hydrochloride 0.5 g l^{-1}, sodium chloride 5.0 g l^{-1}, sodium acetate 3.0 g l^{-1}, agar 0.5 g l^{-1}.

Shahidi-Ferguson Perfringens (SFP) Agar And Overlay.

Sodium metabisulphite and ferric ammonium citrate are present in the medium as indicators of sulphite reduction. *C. perfringens*, a sulphite reducing bacterium, produces black colonies on SFP with overlay. The egg yolk in the media is a substrate for lecithinase, which is released into the surrounding media by most strains of *C. perfringens*. Lecithinase positive colonies have clear halos.

SFP Agar.

Perfringens agar base [OXOID]	500.0 ml
(sterilised and cooled to 50 °C)	
egg yolk	25.0 ml
SFP supplement (SR93) [OXOID]	1 vial
SFP supplement (SR47) [OXOID]	1 vial
Mix well and poor.	

SFP Overlay.

SFP agar omitting the egg yolk.

Perfringens agar base formula; tryptose 15.0 g l^{-1}, soya peptone 5.0 g l^{-1}, Lab - lemco powder 5.0 g l^{-1}, sodium metabisulphite 1.0 g l^{-1}, ferric ammonium citrate
1.0 g l^{-1}, agar 14.0 g l^{-1}, pH 7.6 ± 0.2 .
SFP supplement (SR93) ; 6 mg kanamycin (final concentration 12 mg l^{-1}).
SFP supplement (SR47) ; 15 000 U polymixin B sulphate (final concentration 30 000 U l^{-1}).

SOC Medium

Bacto-tryptone	20.0 g l^{-1}
Yeast extract	5.0 g l^{-1}
NaCl	0.5 g l^{-1}

Autoclave, then prior to use add 1 ml each of filter sterilized $MgCl_2/MgSO_4$ solution* and 2 M glucose solution per 100 mls of basic medium.

*$MgCl_2/MgSO_4$ solution made up to 100 ml in RO water then filter sterilized.

$MgCl_2$ [FISONS]	9.5 g 100 ml^{-1}
$MgSO_4$ [FISONS]	12.0 g 100 ml^{-1}

Stabs Medium.

Used for saving *E. coli* strains, at room temperature and eliminating the need for sub-culturing every few weeks.

Nutrient broth	8.0 g l^{-1}
L-cystine	0.003 g l^{-1}
Agar [DIFCO]	8.0 g l^{-1}

I. B. Tris Based Buffers

Stock Solutions

Tris-HCl.

A selection of tris-HCl stock solutions of various molarities and pH were made up as follows; Tris base (tris (hydroxymethyl) methylamine) [FISONS] was dissolved in a small volume of RO water and the pH adjusted with concentrated hydrochloric acid. It was then made up to the full volume and sterilised.

Sodium EDTA.

Sufficient Na EDTA (diaminoethanetetra acetic acid disodium salt) [FISONS] for a 0.5 M solution was weighed into a beaker, suspended in a small amount of RO water and stirred continuously. Concentrated sodium hydroxide solution was added gradually until pH 8.0 was reached and the remaining undissolved compound went into solution. The volume was corrected with RO water prior to sterilisation.

Buffers

Tris-acetate-EDTA (TAE).

Used in agarose gel electrophoresis of nucleic acids

	(20 X Stock)
Tris base [FISONS]	96.8 g l^{-1}
Na EDTA [FISONS]	7.5 g l^{-1}
Sodium acetate [FISONS]	13.6 g l^{-1}
Dissolved in 200 ml of RO water.	
Glacial acetic acid [FISONS]	33.8 ml l^{-1}
Made up to 1000 ml with RO water.	

Tris-borate-EDTA (TBE).

An electrophoresis buffer used with acryalamide gels e.g. as in DNA sequencing.

	(5 X Stock)
Tris base [FISONS]	54.0 g l^{-1}
Boric acid [BDH]	27.5 g l^{-1}
0.5 M Na EDTA	20.0 ml

Tris-EDTA (TE).

DNA was frequently dissolved in TE buffer.

Tris-HCl pH 8.0	10.0 mM
Na EDTA	0.1 mM

Tris-EDTA-sodium chloride (TES).

Used as an hyaluronidase buffer during the extraction of plasmids from *C. perfringens* [*Mahony et al.*, 1986].

Tris-HCl pH 8.0	0.05 M
Na EDTA	0.005 M

I. C. Bacterial Strains

E. coli Strains

The strains were either bought in as transformation competent cells or were maintained in stab cultures.

E. coli **JM107** [*Yanisch-Perron et al.*, 1985]

supE44 endA1 hsdR17 gyrA96 relA1 thi mcrB Δ(lac-proAB)

F' *[traD36 proAB⁺ lacIᵠ lacZΔM15]*

E. coli K12 strain JM107 is a relaxed mutant i.e. starvation of amino acids does not cause it to undergo the stringent response because it has a mutation in the stringent factor gene, *relA1* [*Gallant*, 1979]. However it will only grow on minimal media that is supplemented with thiamin because the *thi* mutation makes it auxotrophic for this vitamin. This strain is readily transformed because it has an r_K^- m_K^+ phenotype. The *hsdR17 (h*ost *s*pecificity for *D*NA) mutation in the endonuclease subunit *(R)* of *Eco* K, a type I restriction enzyme, prevents restriction of transfected DNA. There are no mutations in the corresponding genes for the methylase (*hsdM* and *hsdS*) so transforming DNA undergoes normal K-modification. The strain has also been found to contain a mutation at the recently discovered *mcrB* loci (*m*ethylated *c*ytosine *r*estriction) [*Raleigh et al.*, 1988], rendering it deficient in the restriction of DNA containing 5-methylcytosine at the dinucleotide sequence 5'-GC-3'. JM107 is lac⁻ because of the *lac-proAB* deletion from the chromosome. The *lacZΔM15*, deletion mutation on the F' episome, which encodes a defective β-galactosidase (it lacks amino acids 11-41) enables the selection of lac⁺ transformants via the α-complementation system. The episome is maintained because it carries the *proAB*⁺ genes which complement the proline auxotrophy caused by the *lac-proAB* deletion from the host chromosome. Analysis of the transforming DNA is aided by the *endA1* mutation, which means that extraction of cloned DNA is not hampered by endonuclease A degradation. *gyrA96* is a mutation in *gyrA* one of two genes, *gyrA* and *gyrB*, needed to make DNA gyrase. The enzyme is a DNA dependent ATPase composed of two gyrα subunits, the component responsible for causing the relaxation of negative supercoils by a breakage-reunion action, and two gyrβ polypeptides. DNA gyrase is needed for DNA recombination so inactivation of the α subunit (*gyrA96*) renders the strain rec⁻ which means that DNA constructs do not undergo rearrangements on entering the cell. The strain is particularly useful for cloning potentially toxic genes because it has the mutant *lacI* gene, *lacIᵠ*. This gene over-expresses the repressor for the lactose operon, reducing

transcription from the *lacZ* promoter, a promoter often used to control expression of foreign genes. The amber suppressor mutation *supE44* inserts glutamine at the UAG codon. It confers on the strain the ability to support the growth of vectors, such as the bacteriophage M13 vectors, which were developed under an old National Institute for Health (NIH) recommendation for cloning vectors to carry amber mutations. The *traD36*, which suppresses conjugal transfer of F factors, is another relic of NIH guidelines.

***E. coli* DH5α [GIBCO BRL]**

supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1

E. coli DH5α is a similar strain to JM107 in that the *ϕ80 lacZΔM15* means it also can be used in the α-complementation screening method. The strain is more deficient in its recombination capabilities because it has a defective *recA* gene, the product of which is essential for all homologous recombination. M13 constructs also undergo fewer deletions when propagated in *recA*$^-$ cells [*Yanisch-Perron et al.*, 1985].

***E. coli* EPICURIAN COLI ® XL1-BLUE [STRATAGENE]**

recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 λ- lac-

F' [proAB$^+$ lacI^q lacZΔM15 Tn10 (tet^R)]

The strain was supplied in the form of competent cells with high efficiencies of transformation (1×10^9 transformants μg^{-1} pBR322 DNA) when used according to the protocol provided. They were stored at -70°C (manufacturers specifications) to prevent loss of efficiency.

XL1-BLUE cells are similar to JM107 but have an additional uncharacterised mutation which enhances α-complementation and also the F' episome carries the Tn*10* transposon which confers a tetracycline resistance phenotype on the host and provides an additional means of maintaining the F' episome.

***E. coli* EPICURIAN COLI ® SURE™ [STRATAGENE] [*Greener* 1990]**

mcrA Δ(mcrBC-hsdRMS-mrr)171 supE44 thi-1 λ- gyrA96 relA1 lac- recB recJ sbcC umuC::Tn5 (kan^r) uvrC F' [proAB$^+$ lacI^q lacZΔM15 Tn10 (tet^R)]

The strain was supplied in the form of competent cells with high efficiencies of transformation (1×10^9 transformants μg^{-1} pBR322 DNA) when used according to the protocol provided. They were stored at -70°C (manufacturers specifications) to prevent loss of efficiency.

E. coli SURE™ are very useful for the cloning of 'unclonable' DNA. DNA molecules which are likely to undergo degradation, rearrangement and/or deletion are those able to form non-standard secondary and tertiary structures

such as Z-DNA (the theoretical left handed helices that may occur at poly $\left\{\frac{GC}{CG}\right\}$ and poly $\left\{\frac{AC}{TG}\right\}$ sequences), cruciforms (due to long inverted repeats) and bent DNA (another theoretical topological change that occurs at polyA tracts) [*Kitchen et al.*, 1986].

Methylated or hemimethylated DNA is not degraded because the *mcrA* mutation and the *(mcrBC-hsdRMS-mrr)171* deletion render this strain completely devoid of any DNA restriction/modification abilities. Strains which are Mcr$^+$ (*m*odified *c*ytosine *r*estriction) restrict methylated DNAs, in particular those which contain 5-methylcytosine. The *mrr* system [*Heitman & Model*, 1987] restricts DNA containing methylated adenine. Whilst *hsdRMS* are the genes for the restriction (*R*), methylation (*M*) and specificity (*S*) subunits of the *Eco* K type I restriction enzyme. [*Bickle*, 1982; *Wood*, 1966].

The *uvrC* mutation occurs in an *u*ltra *v*iolet *r*adiation induced gene which encodes a subunit of the uvrABC endonuclease, the enzyme responsible for initiating the excision repair of damaged DNA. The uvrABC complex is thought to recognise helix-distorting lesions such as pyrimidine dimers. Other helix-distortions that result from the DNA base sequence, such as long inverted repeats may also activate the enzyme since these types of sequences suffer fewer deletions when cloned into a *uvrC* mutant. The *umuC* mutation suppresses the mutagenesis that occurs as a function of the error prone DNA polymerase which is part of the *recA* induced SOS response. An increased sensitivity to UV, an undesirable result of the *uvrC* mutation, is probably alleviated to some extent by the *umuC* mutation.

The *recB* mutation abolishes 95.5% of the homologous recombination in *E. coli* which occurs via the recBCD pathway. It does not however block any recombination that occurs via the recE and recF alternate recB independent pathways. These are both blocked by the *recJ* mutation. The *recJ* gene encodes a 53 kDa single-strand DNA exonuclease. Mutations at the *sbcBC* (suppression of rec*BC* mutants) loci alleviate, via intergenic suppression, the reduced viability associated with the deficiencies in the recombination-repair pathways of *recB*$^-$ and *recC*$^-$ strains restoring recombination via the recF pathway to near wild type levels. The *sbcC* locus in this strain has been inactivated in order to prevent the restoration of recombinational proficiency. According to the manufacturers the *sbcC* and *recJ* gene products are involved in the deletion of cloned Z-DNA sequences. [Reviews: *Smith*, 1988; *Clarke & Low*, 1988]

A wide range of cloning vectors can be used with *E. coli* SURE™. The *recA*$^+$ genotype is necessary for the resolution of products of the θ replication of *red*$^-$ *gam*$^+$ bacteriophage λ vectors into the closed circular templates of rolling

circle replication. Many bacteriophage λ vectors are *red*⁻ *gam*⁻ when the stuffer fragment is removed. The *recB* mutation prevents the *recBC* exonuclease degradation of the concatenated linear λ DNA which would normally be inhibited by the *gam* gene product. The strain also carries the uncharacterised mutation present in the XL-1 BLUE strain which enhances α-complementation.

C. perfringens Strains

All strains were maintained in cooked meat media (OXOID). Storage temperature was initially 4°C but on the advice of a colleague the cultures were removed to a new location at room temperature.

The National Collection of Type Cultures and the F strains were all isolated during confirmed incidents of *C. perfringens* food poisoning and were a gift from Dr J. L. Smart, Institute of Food Research, Bristol Laboratory. Dr Smart also provided the L strains that were isolated from lamb carcasses [*Skjelkvåle et al.*, 1979; *Smart et al.*, 1979]. The PHL strains were isolated during *C. perfringens* food poisoning outbreaks and were obtained from the Food Hygiene Laboratory, Central Public Health Laboratory, London. The enterotoxin negative type A strain (2498/77), the two type C strains (5381 and 5383) and the type D strain were kindly supplied by Dr P. E. Granum, Department of Food Hygiene, Norwegian College of Veterinary Medicine, Oslo.

All strains were Gram-positive, non motile rods that were confirmed as *C. perfringens* by their ability to reduce nitrate, liquefy gelatin and ferment lactose.

Nitrate Reduction

BHIN (BHI containing 0.1% (w/v) potassium nitrate) was inoculated and incubated anaerobically at 37°C for 72 hours. The presence of nitrite was detected by the production of a red precipitate on the addition of 0.8% (w/v) suphanilic acid in 5 M acetic acid followed by 0.6% (w/v) N, N-dimethyl-α-naphylamine in 5 M acetic acid. Zinc dust was added to nitrate reduction negative (colourless) cultures to reduce any remaining nitrate and confirm that nitrate reduction has not occurred; since a colourless reaction would also result if the nitrite was further reduced to hydroxylamine by nitrite reductase [*Sekiguchi et al.*, 1987].

Gelatin Liquefaction and Lactose Fermentation

Gelatin hydrolysis was detected on a modified (12 g l^{-1} agar) LG medium [*Hauschild & Hilsheimer*, 1974a and b]. Inoculated plates were incubated anaerobically at 37°C for 48 hours. Zones of gelatin hydrolysis around the colonies were detected by the addition of saturated ammonium sulphate solution. Acid production due to lactose fermentation was detected by a pH indicator which at low pH produced yellow halos around the colonies.

Motility

Cells grown in BHIN for the nitrate reduction assay were examined for motility using phase contrast microscopy [*Hauschild & Hilsheimer* 1974b; *Smart et al.*, 1979]

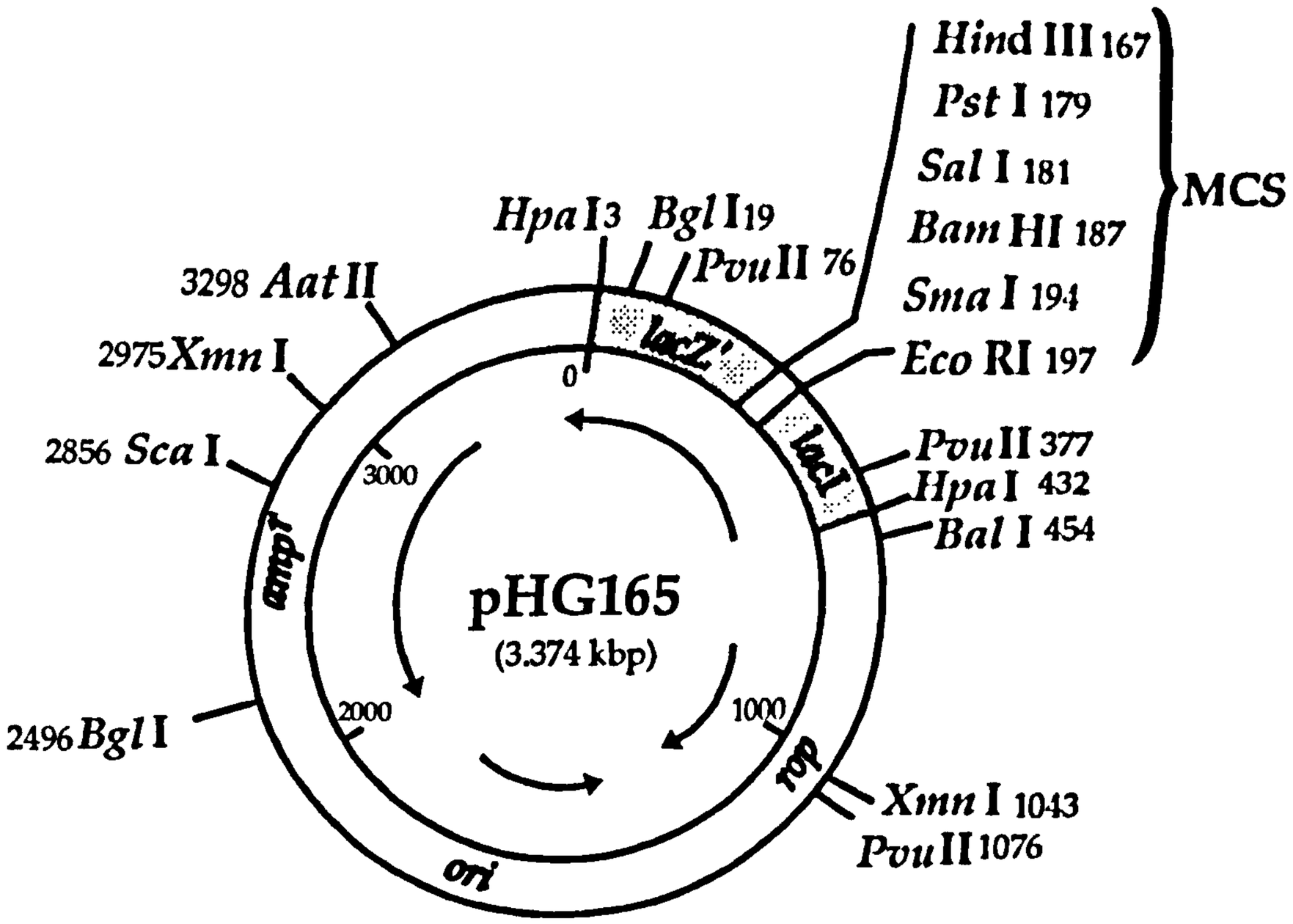
C. perfringens Strains

Chapter	Strain Designation	Year & Place of Isolation	Heat Resistance; Haemolysis	Toxin Type	Serotype	Apparent Size (kbp) of Plasmid Bands
	NCTC 8239	**1952 salt beef**	**HR; NK**	**A (fp)**	**3**	**none**
6,7	NCTC 8235	1951 stew	HR; NK	A (fp)	8	6.6; 5.9
7,9	NCTC 8449	1952 steamed lamb	NK	A (fp)	2	none
6,7	NCTC 8679	1952 faeces	HR; NK	A (fp)	6	6.2
6,7	NCTC 10612	1968 faeces	HR; NK	A (fp)	37	15.5; 7.5; 7.0
7,9	NCTC 10613	1968 Minced beef	NK	A (fp)	15	none
6,7,9	NCTC 10239	1961 rissoles	HR; NK	A (fp)	12	6.0
6,7	NCTC 10240	1959 chicken	HR; NK	A (fp)	13	7.9
6	F611	IFR	HR; H^-	NK (fp)	13	7.8; 6.3; 5.4; 2.5
6,7,9	F1415	IFR	HR; H^+	NK (fp)	29	6.4
6,7	F1785	IFR	HR; H^+	NK (fp)	38	1.0
6	F3419	IFR	HR; H^-	NK (fp)	7	6.6
6	F3417	IFR	HR; H^-	NT (fp)	1	6.2
6	747 / 85	1985 PHL	NK	NK (fp)	3	24.5; 9.0; 5.5
6	509 / 86	1986 PHL	NK	NK (fp)	3	9.0; 6.6; 3.8
6	446 / 86	1986 PHL	NK	NK (fp)	8	no plasmids
6	3040 / 86	1986 PHL	NK	NK (fp)	47	8.0; 5.5; >1.0
6	1032 / 86	1986 PHL	NK	NK (fp)	52,67	1.0
6	73 / 86	1986 PHL	NK	NK (fp)	1	24.5; 9.0; 6.2
6	39 / 86	1986 PHL	NK	NK (fp)	33,62	23.0; 10.0; 6.2; 3.9
6	3049 / 86	1986 PHL	NK	NK (fp)	3/4	29.0; 6.5
7	L4. 1	1978 lamb carcass	NK	NK	2, 3/4, 38, 68	none
7	L5. 4	"	NK	NK	41	18.0; 8.1; 5.5; 3.9
7	L24. 2	"	NK	NK	25	6.5; 4.3; 3.3; 2.3
7	L53. 1	"	NK	NK	61	none
7	L60. 2	"	NK	NK	14	none
7	L99. 4	"	NK	NK	3, 4	8.0; 5.8; 4.0; 3.8 3.2; 2.1; 1.8
7	L100. 4	"	NK	NK	30	5.2; 3.4
7,9	2498/77	[*Skjelvåle et al.*, 1979]	NK	A	NK	NK
7,9	5381	P. E. Granum	NK	C	NK	NK
7,9	5383	"	NK	C	NK	NK
7,9	755	"	NK	D	NK	NK

NCTC = National Collection of Type Cultures; IFR = Institute of Food Research, Bristol Laboratory; PHL = Food Hygiene Laboratory, Central Public Health Laboratories, London; L strains obtained from Dr. J. L. Smart (IFR)[*Smart et al.*, 1979]; P. E. Granum = provided by Dr. P. E. Granum (Norwegian College of Veterinary Medicine, Oslo); (HR = heat resistant; H = haemolysis; A, C, D = toxin types A, C or D; (fp) = associated with a food poisoning outbreak; NK = not known.

I. D. Cloning Vectors

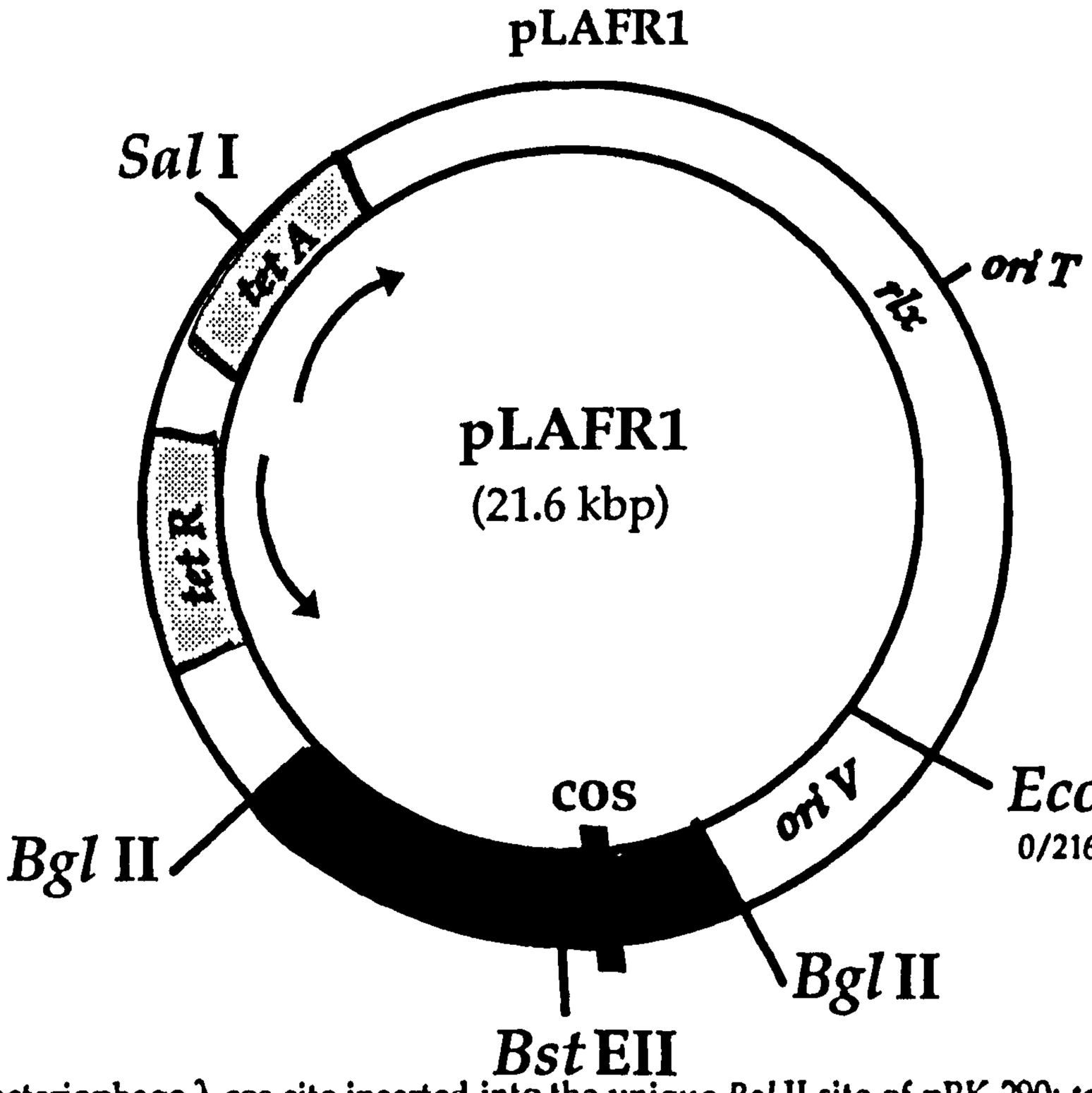
pHG165



MCS = multiple cloning site; *ori* = Col E1 origin of replication; *lac Z'* = 5' end of the *E. coli* β galactosidase gene; *lac I* = *lac Z'* operator region; *amp*^{r} = a β-lactamase gene; *rop* = gene product, ROP, involved in plasmid replication.

The low copy number plasmid vector pHG165 [*Stewart et al.*, 1986], derived from pUC8 [*Viera & Messing*, 1982], was employed for most of the cloning experiments. The high copy number (500-700 per cell) pUC class of cloning vectors [*Viera & Messing*, 1982; *Norrander et al.*, 1983] permit histochemical identification of recombinant clones using the α-complementation system (Chapter 3). The copy number of plasmids such as pHG165, which carry the pMB1 or Col E1 replicon is regulated at the initiation of DNA replication stage. Replication is initiated by a 555 nucleotide RNA primer, produced by RNase H processing of RNA II. The processing of RNA II is inhibited by interaction with an antisense RNA molecule, RNA I. This interaction is aided by the *rop* (*rom*) gene product ROP, a 63 residue polypeptide, by regulating the rate of folding of the 3' end of RNA II and whence the time available for interaction with RNA 1. The significantly increased copy number of pUC plasmids compared to pBR322 is due to unregulated initiation of DNA replication because they lack *rop* gene [*Stueber & Bujard*, 1982; *Twigg & Sherrat*, 1980] and they carry a mutation (G →A) [*Sambrook et al.*, 1989] one base upstream of the transcription initiation site for RNA 1 which results in truncated transcripts which presumably do not interact efficiently with RNA

II [*Sambrook et al.*, 1989]. High copy number plasmids are useful for obtaining large amounts of cloned DNA and cloned gene product. The high copy number can however be disadvantageous if for instance the DNA cloned into the polylinker carries toxin genes. The concomitant high copy number of the *lacI* regulatory region of *lacZ'*, or the inserted DNA, dilutes the repressor molecules resulting in functionally constitutive expression even in *lacI*^{qts} host strains. In pHG165 the control of plasmid DNA replication was recovered and copy number restored to near that of pBR322 (15-20 per cell) by the reintroduction of *rop*.



cos = bacteriophage λ cos site inserted into the unique *Bgl* II site of pRK 290; *tet R* and *tet A* = tetracycline regulatory and resistance genes, respectively; *ori T* and *rlx* = origin of transfer and the *cis*-acting region necessary for conjugal transfer; *ori V* = RK2 origin of replication.

pLAFR1 [*Friedman et al.*, 1982] is a derivative of the broad host range vector pRK290 [*Ditta et al.*, 1985]. A bacteriophage λ cos site was inserted into the unique *Bgl* II site converting the mobilizable RK2 replicon in to a cosmid vector with a unique *Eco* RI cloning site [*Friedman et al.*, 1982]. The low copy number plasmid RK2 belongs to incompatability group P where plasmid replication is controlled by the binding of a repressor protein, TrfA, to multiple repeats of DNA sequences, iterons, [*Nordström*, 1990]. pLAFR1 confers tetracyline resistance on the host but it does not have a system to enable the identification of clones containing recombinant plasmids e.g. an insertional inactivation system.

I. E. Oligonucleotide Probes and Primers

Synthetic oligonucleotides were synthesised by phosphite triester chemistry using nucleoside-3'-*O* (*N,N*-dialkylamino) phosphoramidites as monomers and highest quality acetonitrile as the principal solvent. The 3' protected nucleosides were attached to a glass bead support and the typical steps in phosphoramidite chemistry i.e. removal of 5'-OH protecting groups, coupling, oxidation and capping were automated [BIO-SEARCH]. Oligonucleotides were recovered using the recommended protocol and stored at -20°C in TE.

APPENDIX II
DNA EXTRACTION METHODS

Plasmid DNA Isolation

E. coli Large Scale Plasmid DNA Preparations

Large scale plasmid DNA extractions were prepared and purified from mid exponential phase cultures (grown at 37°C in 500 ml LB) either according to the method of Guerry *et al.* [1973] or using the QIAGEN [© DIAGEN] protocol. For both methods cell lysis was achieved by subjecting lysozyme weakened cells to Triton-X treatment. Cell debris and high molecular weight DNAs were removed from the lysate by centrifugation. The purified plasmid DNA was recovered using the © DIAGEN protocol by passage of the cleared lysate down a QIAGEN column. Higher quality covalently closed circular DNA was obtained by equilibrium centrifugation of the crude plasmid preparation in CsCl-ethidium bromide gradients [*Guerry et al.*, 1973]. The concentrations of plasmid DNA solutions were quantified by comparison, on an 0.8% agarose gel, to DNAs of known concentration.

E. coli Small Scale Plasmid Preparations

Crude plasmid DNA extractions for rapid restriction enzyme analysis were prepared using the alkaline lysis method [*Ish-Horowicz & Burke*, 1981].

C. perfringens Plasmid Extractions

A modification of the Mahony method [1986] was used to isolate the plasmids from *C. perfringens* cultures.

a) Cells were grown anaerobically, at 37°C in 2.0 ml BHI overnight.

b) Cells were pelleted by microcentrifugation for 2 minutes.

c) Cells, resuspended in 90 µl TES buffer (Appendix I) by vortexing, were incubated at 37°C for one hour in 100 µl of hyaluronidase (0. 1 mg ml^{-1} in TES buffer).

d) Pelleted cells (2 minutes microcentrifugation), resuspended in 19. 8 µl tris-sucrose buffer (Appendix I) and 2.2 µl (final concentration 50 mg ml^{-1}) of freshly prepared lysozyme [Sigma] by vortexing, were incubated for a further 30 minutes at 37°C.

e) The activity of nucleases was reduced by the addition of 3.2 µl tris-EDTA (Appendix I).

f) Lysis was achieved by adding 25 µl sarcosyl buffer (Appendix I) and vortexing followed by incubation on ice until a cleared lysate was obtained (about 30 minutes).

g) The crude preparation was extracted twice with TE equilibrated phenol then once with ether.

h) RNA was removed by incubation at 45°C for one hour with 1 mg ml^{-1} RNase A [Sigma] (Treated to remove nucleases according to *Maniatis et al.* [1982]).

C. perfringens Chromosomal DNA Extractions

Procedure One

Chromosomal DNA for the production of a genomic library was isolated from *C. perfringens* strain NCTC 8239 using the procedure described by Okita *et al.* [1981] with an acetone pre-wash [*Heath et al.*, 1986] to aid the SDS induced cell lysis. Cells were cultured overnight, anaerobically at 37°C. The culture medium RCM, which was filtered to facilitate efficient cell pelleting and washing, was later substituted with BHI which improved cell recoveries. Although cell lysis was not consistently achieved even with these modifications.

Very poor quality chromosomal DNA was obtained using this method. It was always sheared to a greater or lesser extent. Preparations with very little sheared DNA were resistant to restriction enzyme digestion. Phenol:chloroform treatments employed to alleviate this problem succeeded only in shearing the DNA. Presumably the DNA was closely associated with proteins rendering it impervious to the actions of the restriction endonucleases and causing breakage of the DNA when removed by phenol extraction.

High levels of DNA packaging would afford the organism reasonable protection against the DNA damaging effects of oxygen free radicals (Chapter 1).

Procedure Two

Recloning and Southern blotting experiments used *C. perfringens* chromosomal DNA isolated from 100 ml BHI overnight cultures, prewashed with 2% NaCl [Personal communication P. E. Granum], using a scaled up GES (guanidium thiocyanate; EDTA; sarkosyl) method [*Pitcher et al.*, 1989].

APPENDIX III
DNA MANIPULATIONS

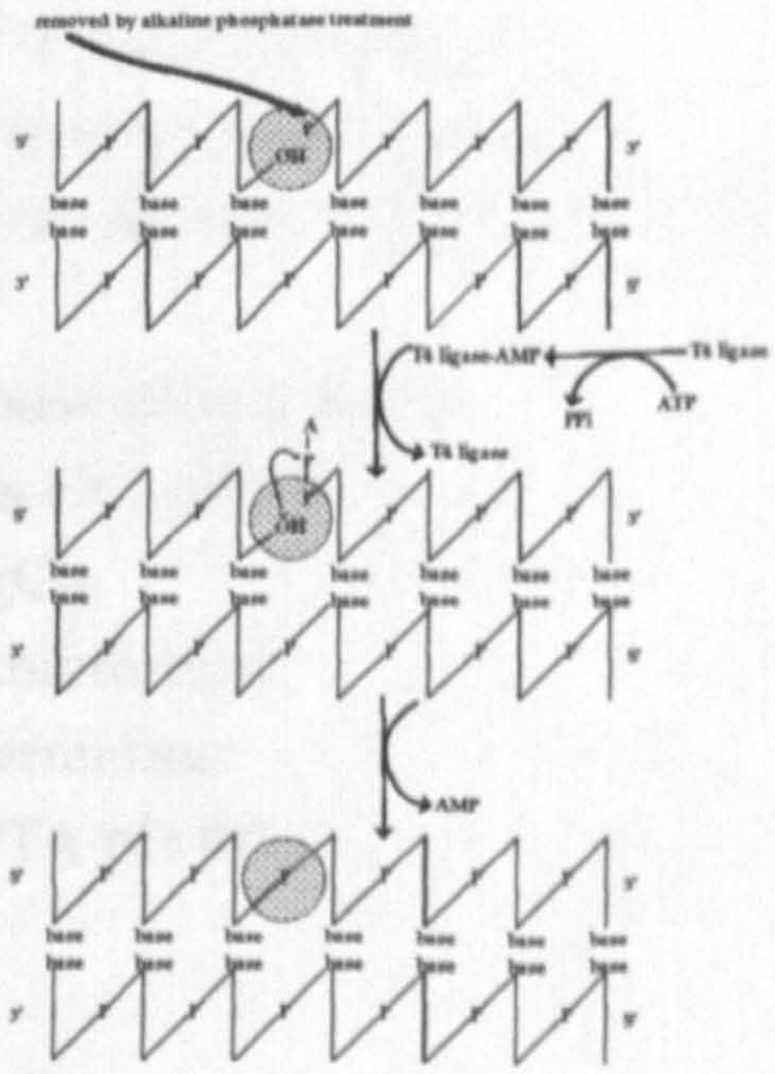
Restriction Enzyme Digests

Restriction enzymes were initially purchased from various suppliers [Pharmacia; Boehringer Mannheim; Amersham] and used either with the buffers supplied or with buffers made according to the particular manufacturers instructions. During the later stages of the project all enzymes were purchased from Pharmacia and used as directed, with the one-phor-all buffer supplied. Digests were performed using the assumption that 1 unit of restriction enzyme digests 1 µg of DNA to completion in one hour under optimum reaction conditions. A minimum reaction volume of 15 µl was employed bearing in mind that the enzyme activity may be inhibited by the glycerol in which it is supplied, if it constitutes more than 0.1 volume of the final reaction mixture.

If necessary the restriction enzymes were either heat inactivated according to the suppliers specifications or were removed from the reactions by phenol:chloroform extraction.

Ligation Reactions

DNA molecules were ligated at 16°C using T4 DNA ligase [Amersham] in the buffer supplied or T4 DNA ligase [New England Biolabs] in 1 X linker kinase buffer (5 X stock stored at -20°C). Self ligation of vectors was prevented by treatment with calf intestinal alkaline phosphatase (CIP) [Pharmacia]. The CIP catalyses the removal of the 5'-phosphate residues which are a vital substrate for the ligation reaction. Dephosphorylation reactions were carried out in one-phor-all buffer according to the manufacturers directions. CIP was heat inactivated and removed by phenol:chloroform extraction.



5 X Linker Kinase

Tris-HCl pH 7.6	330 mM
Spermidine	5 mM
Neutralised ATP	5 mM
Dithiothreitol	75 mM
Gelatin	1 mg ml^{-1}

DNA Labelling

End Labelling

End labelling of oligonucleotides probes was facilitated by the phosphatase activity of T4 polynucleotide kinase [Boehringer Mannheim]. The enzyme catalyses the phosphorylation of the 5' terminus (synthetic oligonucleotides have both 3' and 5' terminal hydroxyl groups) with a radiolabelled γ-phosphate from [γ-^{32}P] dATP. The kinase activity of the enzyme catalyses (in the presence of excess ATP and ADP) the exchange of 5' terminal phosphomonoester groups, of ssDNA or DNA molecules with 5' overhangs, with the γ-phosphate of ATP. The incorporation was stimulated by the spermidine in the reaction buffer, it also inhibited any nucleases that may have been present.

The labelling reactions were set up as shown below. Then incubated for one hour at 37°C. Unincorporated nucleotides were removed by chromatography by passing through a Sephadex G-25 column [Pharmacia]. Percentage incorporation values were always estimated, using a hand held radiation monitor, at greater than 80%.

Reaction Conditions

Oligonucleotide (0.5 µg $µl^{-1}$)	5 µl
PNK buffer (10 X stock)	5 µl
[γ-^{32}P] ATP (37 MBq ml^{-1})	6.5 µl (0.24 MBq)
T4 polynucleotide kinase	1.5 µl (12 U)
Sterile distilled water up to a final volume of 25 µl	

10 X Polynucleotide Kinase (PNK) Buffer

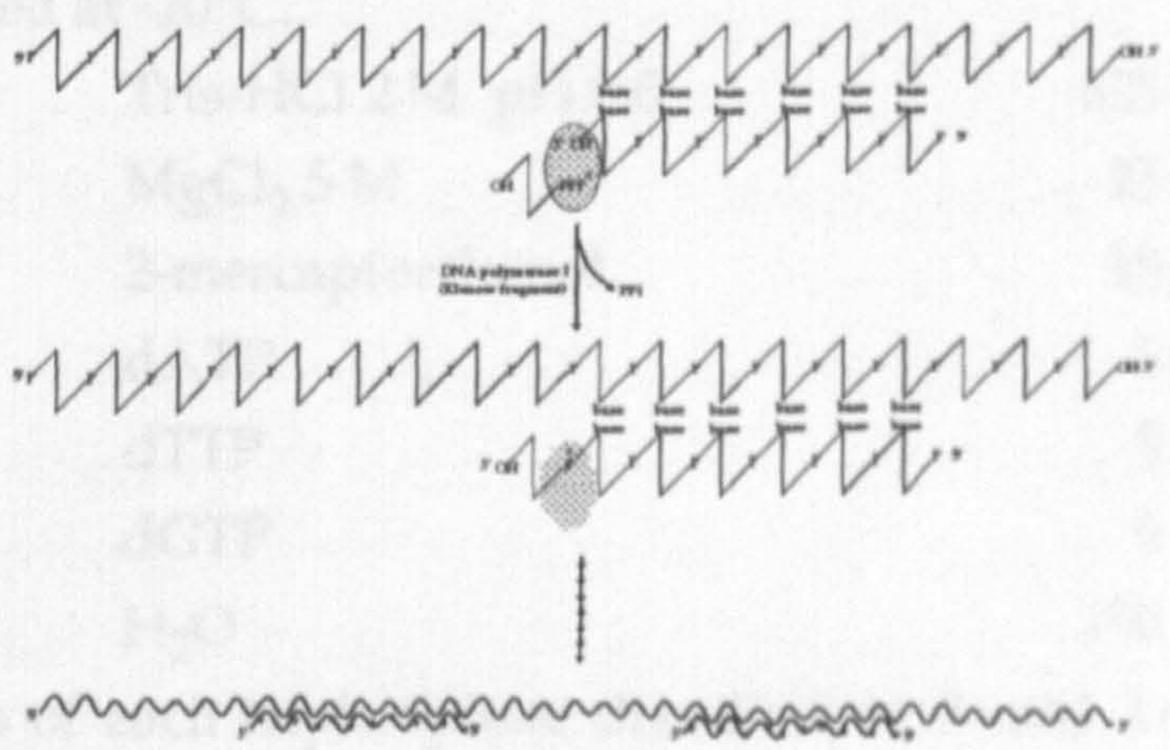
Tris-HCl pH 7.6	0.5 M
$MgCl_2$	0.1 M
Dithiothreitol	50 mM
Spermidine	1 mM
EDTA pH 8.0	1 mM

Random Primer Labelling

Use of the multiple primer DNA labelling reaction [*Feinberg & Vogelstein*, 1983 and 1984] produces probes with very high specific activities. Net DNA synthesis may also occur which means that very little template DNA is required.

The reaction consists of multiple sites of DNA synthesis initiated from short oligonucleotide primers hybridized to the single stranded template. The primers are heterogenous in sequence and therefore hybridize to many sites along the template. They can be derived by digestion of complex dsDNA, such as salmon sperm DNA, with DNase I [*Feinberg & Vogelstein*, 1983] or by the random generation of hexanucleotides on an automated DNA synthesizer. The DNA polymerase used is either the Klenow fragment of *E. coli* DNA-dependent DNA polymerase I for DNA templates or for RNA templates, an RNA-dependent reverse transcriptase.

DNA Synthesis Catalysed by the Klenow Fragment of *E. coli* DNA Polymerase I



Two reaction protocols were employed during the course of this studentship. For both protocols the DNA template was obtained from restriction enzyme digestion of CsCl purified plasmid DNA. The digest was separated by electrophoresis in a 0.6% low melting point agarose gel. The requisite band was excised, suspended in 1.5 volumes of water and boiled for 7 minutes. The fragments were then either stored at -20°C or placed at 37°C for 10 minutes prior to use in a labelling reaction. Stored fragments were reboiled for 3 minutes and kept at 37°C for 10 minutes immediately before utilization in a labelling reaction. The radiolabel was [α-^{32}P] dCTP (specific activity $\cong$ 1.11×10^4 GBq (3000 Ci) mmol^{-1} (3.4 pmol μl^{-1}; 0.37 MBq (10 μCi) μl^{-1}) [Amersham]. Unincorporated nucleotides were not removed. Percentage incorporation was estimated by comparative monitoring of two reaction samples (0.001% of the reaction volume) air dried onto nitrocellulose filters. Sample one was precipitated with 5% trichloroacetic acid (TCA) then both

samples were rinsed with water and air dried. The counts obtained from the TCA precipitated sample *versus* the unprecipitated sample enabled a qualitative opinion of reaction success to be formed. Occasionally reaction products were assayed by PAGE followed by autoradiography to ensure they were labelled and of sufficient length.

Protocol One

Reaction carried out overnight at room temperature.

H_2O	up to a final volume of 30 µl
OLB	6 µl
BSA	1.2 µl
DNA	up to 25 ng (minimum 5 ng)
[α-^{32}P] dCTP	3 µl
Klenow	1.2 µl (1 unit/1 µl)

OLB

Made from a mixture of solutions A:B:C in the ratio of 2:5:3.

Solution A: Stored at -20°C.

Tris-HCl 2 M pH 8.0	625 µl
$MgCl_2$ 5 M	25 µl
2-mercaptoethanol	18 µl
dATP	5 µl
dTTP	5 µl
dGTP	5 µl
H_2O	350 µl

(0.1 M solutions of each triphosphate dissolved in 3 mM Tris-HCL, 0.2 mM EDTA, pH 7.0).

Solution B: Stored at 4°C.

2 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) titrated to pH 6.6 with NaOH.

Solution C: Stored at -20°C.

Hexadeoxyribonucleotides [Pharmacia] evenly suspended in 3 mM Tris-HCl, 0.2 mM EDTA, pH 7.0.

BSA

Enzyme grade BSA (BRL) 10 mg ml^{-1} in H_2O. (Equivalent amount of BSA sometimes used from a molecular biology grade [SIGMA] 4 mg ml^{-1} solution).

Protocol Two

The Amersham Multiprime Labelling Kit RPN. 1600Z using the standard Multiprime DNA labelling protocol but with the DNA in the same quantities and prepared in the same manner as for protocol one.

Agarose Gel Electrophoresis

DNA fragments for restriction enzyme analysis or for purification were separated by electrophoresis in agarose gels.

The rate of migration of DNA through an agarose gel varies with pore size which in turn is determined by the agarose concentration. The pores are formed by hydrogen bonding between the unbranched chains of galactan when dissolved in an aqueous solution (achieved by heating to 90°C). DNA for radiolabelling was purified using 0.6% low melting point agarose [Pharmacia] all other gels were 0.8% agarose NA [Pharmacia].

The two types of gels were made up in 1 X TAE buffer (Appendix I). DNA samples were loaded in gel-loading buffer type III [*Sambrook et al.*, 1989] to ensure that the sample sank to the bottom of the well and to enable the course of the electrophoresis to be monitored. The gels were all electrophoresed using 1 X TAE buffer but at a variety of voltages according to experimental requirements.

The DNA was visualised by staining the gels with ethidium bromide [*Sharp et al.*, 1973], a fluorescent dye which intercalates between the stacked bases of the DNA. The ethidium bromide fluoresces, indicating the positions of the DNA bands, when viewed using UV light transillumination. The gels were photographed onto Ilford FP4 photographic films (ASA 125, DIN 22). Photographs were taken with a 5" Polaroid lens mounted onto a W.5 land camera fixed on an industrial accessory mount. Exposures were for 30 seconds at F/5.6 . Films were processed in Paterson Acutol developer and Ilford Speed fixer according to the manufacturers instructions.

Hind III digests of bacteriophage λ (cIts857) DNA were used on every gel for the construction of calibration curves. The sizes of the restriction fragments were known and the migration distances was measured for each gel. The curves were produce using the assumption that the migration rates of the DNA fragments through the gel are inversely proportional to the $\log_{10}$ of the base pairs [*Helling et al.*, 1974].

Extraction and Purification of DNA from Agarose Gels

For many applications such as cloning the DNA has to be removed from the agarose because contaminants in the gel are liable to inhibit enzymes such as ligases. Highly purified preparations such as the low melting point agaroses may be less of a problem.

Electroelution

Gel containing the desired DNA was excised and transferred to dialysis tubing (prepared according to Sambrook *et al.* [1989]) filled with 1 X TAE. It was then placed in an electrophoresis tank and a high voltage gradient applied across it causing the DNA to move out of the gel and in to the buffer.

Gene Clean™ [STRATAGENE] and Prep A Gene™ [BIO RAD] Kits

(Used according to manufacturers instructions).

The gene clean kit relies on the fact that agarose gels are held together by hydrogen bonds which are easily broken by heat and chaotropic agents, such as KI, releasing the DNA. The DNA can be recovered by virtue of the fact that DNA sticks to glass in high salt concentrations. The ethidium bromide and salt solution can be washed from the DNA by ethanol while it is still attached to the glass (the kit employs glass beads). Elution from the glass requires a dilute buffer. Large DNA fragments <10 kbp may be sheared using this method.

Buffer-X Method

DNA containing agarose slices were cut from the gel and homogenised (to aid diffusion of the DNA molecules from the gel) by gently passing through a 1 ml syringe. The gel was placed in a 35 ml Beckman polypropylene centrifuge tube overlaid with the elution buffer-X and left overnight at 37°C. The gel was removed by centrifugation. The DNA solution was cleaned by phenol:chloroform extraction and ethanol precipitated.

Buffer-X

Ammonium acetate [FISONS]	500 mM
Magnesium acetate [FISONS]	10 mM
EDTA pH 8.0	1 mM
Sodium dodecyl sulfate	0.1%

NACS 52 PREPACK Columns [GIBCO BRL]

Further purification of the DNA was achieved by passing the DNA in an appropriate buffer through a NACS chromatographic column (according to instructions).

Low Melting Point Agarose

As discussed at the start of this section low melting point agarose contains fewer impurities than lower grade agaroses so it was possible to perform manipulations, such as random primer labelling, without extracting the DNA from the gel.

Polyacrylamide Gel Electrophoresis (PAGE)

Agarose gel electrophoresis is unable to resolve DNA molecules less than 1000 bp instead polyacrylamide gels were used. The type of acrylamide gel required varies with the size of the DNA molecules for separation [*Maniatis et al.*, 1975]. The rate of migration through the gel varies with pore size, which unlike agarose gels is determined by both the total acrylamide content (%T= acrylamide + N,N'methylenebisacrylamide (usually referred to as 'bis') measured in g 100 cm^{-3} of gel mixture) and the ratio of acrylamide:'bis'. The pores are produced by the formation of cross-linkages, by 'bis', between acrylamide polymers during the polymerisation reaction. The free radical chain reaction, which causes the polymerisation, is initiated by the generation of radicals. The activation of N,N,N',N'-tetramethylethylethylenediamine (TEMED) by ammonium persulphate produces such a radical.

Urea is incorporated into the gels to ensure that small DNA molecules >1000 bp, such as those generated during DNA sequencing reactions, remain denatured. Denaturation of larger molecules or ssDNA is further aided by the use of 98% formamide in the loading buffer.

PAGE of Oligonucleotides

100 ml of 16% acrylamide solution was required for a 140 mm^2 gel. 40 µl of TEMED was added to 40 ml of the solution and used to pour a plug. When the plug was set 60 µl of TEMED was added to the remainder of the solution and used to cast the gel. The comb was put in place and the gel left to polymerise overnight. The gel was pre-run with 1 X TBE electrophoresis buffer for 30 minutes at 350 V prior to sample loading. 1 µg of oligonucleotide in 98% formamide;10 mM EDTA was loaded on to each well. The sample front indicator dyes bromophenol blue and xylene cyanol were loaded into a separate well in order not to interfere with the migration or visualisation of the oligonucleotides. The gel was run at 350 V until the first dye band had migrated two thirds of the way down the gel. The gel was submerged in ethidium bromide solution (0.5 µg ml^{-1}) for 30 minutes then viewed on a transilluminator. A photograph was taken as for agarose gels.

16% Acrylamide Gel Solution:

Urea[FISONS]	7 M	42.0 g
Tris-borate[BDH]	90 mM	5 X stock
EDTA	2.0 mM	500 mM stock
acrylamide	16.0%	5 X stock
ammonium persulphate	1.0 ml	
water	up to 100 ml	

5 X Acrylamide

Acrylamide [BDH chemicals]	38 g
'Bis' [BDH chemicals]	2 g

Made up to a final volume of 100 ml with distilled water. Stirred until completely dissolved then filter sterilised.

In the later stages of the project a BIO-RAD Mini-PROTEAN II gel system was used according to the protocol provided. The protocol was essentially the same except that the gels were very much smaller and only required 10 ml of solution. They could also be electrophoresed at a higher voltage.

PAGE of Sequencing Reactions

The 8% gel was poured between two glass plates (330 mm X 400 mm) separated by a 0.4 mm plastic spacer. The plates were either taped together or on a gel pouring apparatus. The thermostatic plate was treated with bind silane (γ (methacryloxy)-propyltrimethoxysilane) [Sigma] so that the gel was covalently bound to it. The notched plate was silica coated (Sigma Cote®) to repel the gel and prevent it from sticking to both plates. The wells were formed by a 24 lane sharks tooth comb.

Gels were prerun for 30 minutes with 1 X TBE running buffer. 4 µl of each sample was loaded (always in the order GATC) after denaturing at 80°C for 3 minutes, and electrophoresed at 1200 V, 25 mA until the first dye band had run off the bottom (≅ 2 hours), until the second dye band had run of the bottom (≅ 4 hours) or for a total of six hours. The gel was the fixed in a solution of glacial acetic acid:methanol:water (1:1:8) to remove the urea. Gels that contain urea distort and crack when dried and are tacky to touch so that the autoradiography films stick to them. The gels were blotted dry with tissue paper then dried either at room temperature overnight or at 55°C for 2 hours. Films (Hyperfilm™-βmax. High Performance Autoradiography Film) [Amersham] were exposed for a minimum of 12 hours depending on the activity of the radiolabelled dATP. They were then processed using Ilford Contrast FF developer and Ilford Speed 2600 fixer according to manufacturers instructions.

Bind Silane Treatment

a) Plates were washed thoroughly with 0.1% SDS and rinsed with water.
b) Polished with ethanol.
c) Wiped with 0.5 N NaOH to remove traces of previous gels.
d) Evenly spread with 5 ml of Bind Silane solution.
e) Air dried for 5 minutes then polished with tissue paper.
f) Unbound solution was removed by vigorous wiping with ethanol followed by polishing with dry tissue paper.

Bind Silane Solution.

(Made up fresh every time)

Bind Silane	5 µl
10% acetic acid (diluted in H_2O)	175 µl
Ethanol	5 ml

Sigma Cote® Treatment

a) As above.
b) As above.
c) 5 ml of Sigma Cote® solution was spread evenly over the plates and left to dry.
d) As for c).
e) The plates were polished well with dry tissue paper.

Sigma Cote® Solution. Made up fresh every time.

5% Sigma Cote® in 10 ml of chloroform.

8% Acrylamide Gel for Sequencing

Urea	25.2 g
5 X TBE	12 ml
5 X acrylamide	12 ml

Made up to 60 ml with water and mixed to dissolve.

Polymerisation was initiated by the following additions when everything was ready for gel pouring and after a test polymerisation had proven satisfactory.

10% ammonium persulfate	500 µl
TEMED	70 µl

DNA Sequencing Reactions

All DNA sequences were elucidated using chain termination based sequencing methods. DNA synthesis was initiated from synthetic oligonucleotide primers hybridized to the denatured, CsCl gradient purified [Appendix II], plasmid template. The DNA replication was catalysed by either the Klenow fragment of the *E. coli* DNA-dependent DNA polymerase I [Amersham], avian myelocytomatosis virus (AMV) reverse transcriptase [Boehringer Mannheim] or Sequenase [Pharmacia], the bacteriophage T7 derived DNA polymerase. DNA replication was performed using radiolabelled dATP [α-^{35}S] dATP 370 MBq (10 mCi) ml^{-1} (>3.7 X 10^3 GBq (1000 Ci) mmol^{-1}) [Amersham] as a substrate. Chain termination was effected by the random incorporation into the growing DNA strands of 2'-3'-dideoxyribonucleotide triphosphates [New England Biolabs], which lack both the 2' and the 3' hydroxyl groups, the 3' OH being essential for chain elongation (see diagram with **Random Prime Labelling Protocols**). Four reactions, one each for ddATP, ddTTP, ddGTP and ddCTP generated four heterogenous populations of ^{35}S labelled DNA, the chains in each population terminating with a different dideoxynucleotide. Chains were separated by electrophoresis for two, four or six hours in an 8% polyacrylamide gel.

Klenow and Reverse Transcriptase

Annealing of the Primer and the Template

a) 4 µl (1 µg) of template was denatured for 5 minutes at room temperature by the addition of 1 µl 2N NaOH.

b) 1 µl (52 ng) of primer was added and mixed thoroughly.

c) The reaction was neutralised by 1.5 µl of 3 M sodium acetate pH 4.6 and made up to 9 µl with H_2O.

e) The annealing reaction was then dialysed against TE on a Millipore vswp 0.025 µM membrane for 45 minutes.

f) 11 µl were recovered and used as the substrates for both the Klenow and the reverse transcriptase catalysed reactions.

Reverse Transcriptase Reactions

Carried out essentially as described previously [*Bartlett et al.*, 1986].

a) 4 µl of a sequencing cocktail was added to 1 µl of each of the four dideoxynucleotide reaction mixes.

b) The chain elongation reactions were incubated at 37°C for 20 minutes.

c) 1 µl of the chase solution was added and the incubation continued for a further 20 minutes.

d) The reactions were stopped by 10 µl of stop solution then stored at -20°C until required.

Sequencing Cocktail

Recovered DNA	11 µl
Buffer R	4 µl
[α-^{35}S] dATP	4 µl
Reverse transcriptase	1 µl

Buffer R

0.3 M Tris-Hcl pH8.3
0.312 M NaCl
37.5 mM $MgCl_2$
30 mM dithiothreitol
6 mM spermine (tetrachloride) [Sigma]

ddNTP Mixtures

Prepared according to Bartlett *et al.* [1986] except that the dNTPs [Boehringer Mannheim] were at a concentration of 2.5 mM and the ddATP mix contained 6.1 µl of the New England Biolabs A mixture as opposed to 2.1 µl.

dNTP Chase Solution

Was freshly prepared each time from 14 µl of a New England Biolabs dNTP chase mixture which contained 0.25 mM dATP; 0.25 mM dCTP; 0.25 mM dGTP; 0.25 mM dTTP and 2 µl of reverse transcriptase.

Stop Solution

The chase solution, deionized formamide containing 0.3% xylene cyanol FF; 0.3% bromophenol blue; 0.37% EDTA pH 7.0, was supplied ready prepared by New England Biolabs.

Klenow Reactions

The Klenow sequencing reactions were as described by Williams *et al.* [1986].

a) 3 µl of a sequencing cocktail were added to 2 µl of each ddNTP mix.
b) The reactions were incubated at 37°C for 20 minutes.
d) 1.5 µl of chase solution. Incubation continued for a further 20 minutes.
e) 6 µl of stop solution was added and the reactions stored at -20°C until needed.

Sequencing Cocktail

Recovered DNA	9 µl
10 X sequencing buffer	1 µl
[α-^{35}S] dATP	2.5 µl
Klenow	2 µl (10 U)

Sequencing Buffer
100 mM Tris-HCl pH 7.5; 50 mM $MgCl_2$; 75 mM dithiothreitol supplied by New England Biolabs.

ddNTP Mixtures where as supplied [New England Biolabs].

Chase Cocktail
10 µl of New England Biolabs chase solution plus 3 µl of Klenow.

APPENDIX IV
TRANSFORMATION PROTOCOLS

Heat Shock Induced Transformation of *E. coli* [Lederberg & Cohen 1974]
Pelleted log phase *E. coli* cells, grown at 37°C in 250 ml *LB, were resuspended* in ice cold 100 mM CaCl and incubated on ice for a minumum of one hour and a maximum of over night. 10-20 µl of plasmid DNA was added to 200 µl of the now transformation competent cells and the incubation on ice continued for a further 30 minutes. The cells were then heat shocked at 42°C for 3 minutes and quenched on ice for 30 minutes. Cells were then suspended in LB and incubated at 37°C, 200 rpm for one hour to take account of the phenotypic lag encounted with some selectable markers such as antibiotic selection. Transformants were then selected by plating the cells on selective media (MacAmp for pHG165 and LBtet for pLAFR1).
Protocols supplied with the ready competent cells recquired the addition of 1:10 dilutuion of β-mercaptoethanol (diluted in water) to the cells giving a final concentration of 25 mM. They also recommended the use of SOC, rather than LB, as the nutrient medium during the one hour incubation.

Electroporation Induced Transformation of *E. coli* [Dower et al., 1988]
E. coli cells were made competent for electroporation induced transformation by the method recommended by the manufacturers of the electroporation apparatus [BIO-RAD] using water as the wash buffer. Cells were suspended in 10% glycerol and stored at -70°C until required.
Transforming DNAs were suspended in and dialysed against TE to reduce arcing. Cells were then electroporated according to the electro-transformation procedure described in the BIO-RAD *'Bacterial Electro-transformation and Pulse Controller Instruction Manual'* (Version 1.0).

APPENDIX V
HYBRIDIZATION PROTOCOLS

DNA hybridizations were either as described for capillary (Southern) transfer of DNA and hybridization with formamide [© *Gelman Sciences*, 1987] (Protocol one) or DNA transfer was according to the Hybond N+ [Amersham] protocol with BLOTTO [*Sambrook et al., 1989*] hybridization buffer. The later method employed 0.4 N NaOH as the DNA transfer buffer as opposed to the capillary method which used 10 X standard saline citrate (25 X SSC: 3.75 M NaCl, 0.375 M sodium citrate, pH 7.0). The two methods both employed formamide to reduce the hybrid T_m but the effective probe concentration (Chapter 3) was increased either with dextran sulfate (first method) or polyethylene glycol (BLOTTO method). Non-specific binding sites on the filters were blocked by the first method using a combination of sheared salmon sperm DNA and Denhardt's Solution (100 X Denhardt's solution: 2% ficoll 400, 2% polyvinylpyrrolidone, 2% bovine serum albumin in water) and by polyethylene glycol in the BLOTTO method. The wash buffer for both methods was SSC (various strengths) with 0.1% (w/v) sodium dodecyl sulfate.

Intensifying Screens

The sensitivity of detection of the high energy β particle emitted by ^{32}P labelled probes was enhanced by the use of solid state scintillation i.e. a high density fluorescent 'intensifying screen' was placed on top of the X-ray film. The particles are captured by the screen and reflected as visible light which blackens the film.

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List of Abbreviations

A - adenosine
A - absorbance (1 cm)
aa - amino acid
AMV - avian myeloblastosis virus
Ap - ampicillin
βgal - β-galactosidase
bp - base pair(s)
BSA - bovine serum albumin
C - cytidine
ccc - covalently closed circular
d - deoxyribo
Δ - deletion
dd - dideoxy
DNase - deoxyribonuclease
dNTP - deoxyribonucleoside triphosphate
ds - double strand(ed)
DTT - dithiothreitol
ELISA - enzyme-linked immunosorbent assay
EtBr - ethidium bromide
G - guanosine
IPTG - isopropyl-β-D-thiogalactopyranoside
IS - insertion sequence
kbp - kilobase (S) or 1000 bp
kDa - kilodalton (s)
LB - Luria-Bertani (medium)
MCS - multiple cloning site
N - any nucleoside
NAD - nicotinamide-adenine dinucleotide
NADH - reduced NAD
ORF - open reading frame
ori - origin of DNA replication
p - plasmid
PAGE - polyacrylamide gel electrophoresis
PEG - polyethylene glycol
Pi - inorganic phosphate
PPi - inorganic pyrophosphate
R or r - (superscript) resistance/resistant
rDNA - DNA coding for ribosomal RNA
RNase - ribonuclease
rRNA - ribosomal RNA
S or s - (superscript) sensitivity/sensitive
SD - Shine-Dalgarno (sequence)
SDS - sodium dodecyl sulfate
ss - single strand(ed)
SSC - 0.15 M NaCl/0.015 M Na_3 · citrate pH 7.6
T - thymidine
Tc - tetracycline
Tn - transposon
u - units
U - uridine
UV - ultraviolet
wt - wild type
Xgal - 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
// - denotes plasmid caarier state
:: - novel joint (fusion)
' (prime) - denotes a truncated gene at the indicated site