The Characterisation of a novel *Xanthomonas* Bacteriophage

by

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A dissertation submitted in partial fulfilment of the requirement for the degree of Master of Science in the Department of Microbiology, University of Cape Town.

March 1999

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ACKNOWLEDGMENTS

I wish to sincerely thank my supervisors, Dr. Molapo Qhobela and Assoc. Prof. Edward Rybicki for their advice and guidance during this project. I would like to thank the technical staff of the Microbiology Department, especially Di James for doing a wonderful job of sequencing my clones; Annemarie Clennel and Di de Villiers for making life so much easier by taking care of "the little details". I am grateful to Mohamed Jaffer for his assistance with electron microscopy and photography. Thank you to Lemese Ah Tow for the early morning talks; Eva and Palle for their encouraging and supportive e-mail. The members of the anaerobe unit deserve a special mention for putting up with me spending more time in their lab than in my own during the past year and for occasionally helping me think of "the perfect sentence".

Finally I wish to thank my parents, sisters and brother-in-law for their encouragement and support throughout my years of study.

I acknowledge the financial support of the University of Cape Town as well as the Foundation for Research and Development.

ABSTRACT

During the 1994-95 growing season, an apparently new disease was observed on *Brassica oleracea* var. *capitata* (cabbage) seedlings in nurseries of the province of KwaZulu-Natal, South Africa. Named chocolate spot disease of crucifers because of the characteristic symptoms of dark brown to black lesions with transparent centers, this disease was particularly severe on the more mature leaves of stressed seedlings and resulted in serious economic losses. The causal agent, determined to be a strain variant of *Xanthomonas campestris* pv. *campestris* was found to be associated with bacteriophage-like particles.

The optimal growth conditions for production of the bacteriophage-like particles by the chocolate spot pathogen were determined and a purification protocol developed that yielded particles in a quantity and quality adequate for further analysis. Based on a comparison of the particle and bacterial protein reactions with the antiserum raised against the purified particles, it was concluded that the particles were not vesicles originating from the bacterial membrane, but were therefore most probably of bacteriophage origin.

Physicochemical characterisation showed that the putative phage is a tailless isometric particle with a diameter of 33.45 nm, sedimentation coefficient of 85S and a density in CsCl of 1.347 g/ml. The phage is relatively stable with respect to pH, solvents and at temperatures of 40°C and 60°C. The particles contain 3 major proteins of 94, 32 and 21.8 kDa as well as two minor proteins of 40 and 25.7 kDa, and it has a single-stranded DNA genome.

Biological characterisation of the novel bacteriophage indicates that it is a temperate phage which does not cause visible plaque formation on solid media and is not inducible by the external factors, mitomycin C and ultraviolet radiation. Serological tests showed that the phage is present in all isolates of the chocolate spot pathogen and that similar particles are associated with the *X. campestris* pathovars *aberrans*, *armoraciae*, *campestris* and *raphani*. No serological relationship was detected between the novel phage and the *X. c.* pv. *campestris* phage, RR68. However, the novel phage antibodies recognised 3 proteins of molecular weights 21.8, 13.79 and 12.64 kDa in the *X. c.* pv. *campestris* phage, HT_{3h}. These proteins are localised in the phage capsid. Although phage-like particles were detected in the novel phage host PCB 22 following electron microscopy of ultra-thin sections, immunogold-labelling could not confirm whether the particles were of phage origin.

The single-stranded DNA genome of the phage hybridised with a 53.9 kb extrachromosomal DNA element. Since its size precludes this element being packaged as single-stranded DNA into a capsid 33.45 nm in diameter, it is most probable that this 53.9 kb DNA element is an indigenous plasmid into which the double-stranded form of the phage genome has integrated. However, this could not be confirmed by the results of the nucleic acid hybridisation tests.

The 53.9 kb extrachromosomal element was cloned and several recombinant plasmids sequenced. No typical phage genes were identified. However, fragments of a *Xanthomonas* avirulence (*avr*) gene interrupted by part of a transposon sequence were identified. A 201-262 base region of clone pSSI shared a 98% identity to the 3′ end of a group of *Xanthomonas avr* genes, while 141-311 bases had a high degree of nucleotide similarity to the 5′ end of the *avr* genes. These two regions of *avr* gene similarity which appear to converge, are interrupted by 1259 bases sharing 99% nucleotide identity to the 4864-6126 bp position of the 6938 bp *X. campestris* transposon, ISXC5 and 298 bases with 97% similarity to the 6644-6938 bp region of the transposon. The 1259 and 298 base regions are in turn separated by an unsequenced region of 518 bases. The presence of the *avr* and transposon sequences (usually located on plasmids or the bacterial chromosome) on the extrachromosomal element strengthens the hypothesis that the phage genome is integrated into an indigenous plasmid.

ABBREVIATIONS

BSA	bovine serum albumin
°C	degrees Celsius
CsCl	caesium chloride
EDTA	ethylenediamine-tetraacetic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	kilobase
kDa	kilodalton
KH_2PO_4	potassium dihydrogen phosphate
λ	lambda
M	molar
mA	milliampere
$MgSO_4$	magnesium sulphate
min	minute
ml	millilitre
mM	millimolar
Na_2HPO_4	disodium hydrogen orthophosphate
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanogram
NH_4Cl	ammonium chloride
nm	nanometre
PAGE	polyacrylamide gel electrophoresis
pMol	picomole
pv	pathovar
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
TEMED	N, N, N', N'-tetramethylenediamine

Tris	hydroxymethyl aminomethane
Tris-Cl	hydroxymethyl aminomethane chloride
U	unit of enzyme activity
UV	ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
µg	microgram
µl	microlitre
µm	micrometre

CHAPTER 1

General Introduction

Contents

1.1. Chocolate Spot Disease of *Brassica oleracea* var *capitata*

An apparently new disease was observed on *Brassica oleracea* var. *capitata* (cabbage) seedlings in nurseries of the province of KwaZulu-Natal, South Africa, during the 1994-95 growing season. The disease manifested itself on seedlings, as small water-soaked lesions that expanded to the size of match heads (approximately 3mm in diameter) and became chocolate brown with translucent centres. The symptoms were particularly severe on the more mature leaves of stressed seedlings, and infected seedlings died before reaching transplant maturity (Laing and Qhobela, 1995). Although it is known that symptoms of black rot - a disease of crucifers caused by the pathogen, *Xanthomonas campestris* pv. *campestris* - vary depending upon the plant host and environmental conditions (Hayward, 1993), the symptoms observed in KwaZulu-Natal did not correspond to those caused by *X. campestris* pv. *campestris* or any other known pathogens of cabbage.

A yellow pigmented bacterium, consistently isolated from infected plants, was shown to be the causal agent of the disease. This bacterium was indistinguishable from *X. campestris* and its pathovars. However, SDS-PAGE of its cellular proteins, random fragment length polymorphism (RFLP) analysis of its genomic DNA and FAME (fatty acid methyl ester) analysis showed that the isolates form a group distinct from *X. campestris* pathovars. Similarity ranking and phylogenetic analysis of the 16S rDNA sequence indicated that the causal agent of chocolate spot disease of cabbage is a member of the gamma sub-division of the *Proteobacteria* with *X. campestris* pv. *campestris* as its closest relative. Based on these results and the rules of bacterial nomenclature (Dye *et al.*, 1980), it was concluded that the chocolate spot pathogen does not constitute another pathovar of *X. campestris*, but rather a strain variant of the pathovar *X. campestris* pv. *campestris* (Kariem, 1994).

During the course of the investigation of the bacterial morphology by electron microscopy, it was discovered that all the isolates of the chocolate spot pathogen were associated with bacteriophage-like particles (Figure 1.1; M. Qhobela, unpublished data). No visible plaque formation was observed in cultures of the bacteria on solid media: lack of plaque formation is known to be a distinguishing characteristic of temperate phages engaged in a non-lytic life cycle; thus it was assumed that the particles could in fact be phages.

In the following sections I will give an overview of the genus *Xanthomonas* and the bacteriophages associated with it, as well as discussing bacteriophages with respect to their properties and the role they play in molecular biology.

Figure 1.1. Electron micrograph of PCB 22, an isolate of the causal agent of chocolate spot disease of cabbage. The arrows indicate the bacteriophage-like particles. Magnification: 30 000x. The scale bar represents 300 nm.

1.2. The Bacterial Genus *Xanthomonas*

The genus *Xanthomonas* constitutes a distinct branch in the gamma sub-group of the *Proteobacteria,* a phylogenetic taxon which includes the purple bacteria (Stackebrandt *et al.,* 1988). A combination of biochemical, physiological and molecular characterisation has now led to the recognition of the following six validly described species within the genus *Xanthomonas*: *X. albilineans, X. axonopodis, X. campestris* (type species of the genus), *X. fragariae, X. oryzae* and *X. populi* (Bradbury, 1986; Vauterin *et al.*, 1993; Moore *et al.*, 1997).

All members of the genus *Xanthomonas* are Gram negative, strictly aerobic, non-sporing rods, occurring mostly alone or occasionally in pairs (Dye, 1966). Except for *X. albilineans, Xanthomonas* species produce and are surrounded by the extracellular heteropolysaccharide, xanthan gum, which is manifested in the large mucoid colonies produced on solid media (Van den Mooter and Swings, 1990). A distinguishing feature of this genus is the production of a yellow, water-insoluble carotenoid pigment, called xanthamonadin (Swings *et al.*, 1993). However, several pathovars of *X. campestris* which exhibit features typical of the genus, do not.

produce pigment (Van den Mooter and Swings, 1990). Another characteristic that serves to differentiate *Xanthomonas* from other bacteria is the presence of the three fatty acids, 11:0 iso, 11:0 iso 3OH and 13:0 iso 3OH, which are unique to the genus (Yang *et al.*, 1993).

There is great interest in the genus not only because of the important biotechnological applications of xanthan gum as a result of its unusual physical properties, but also because bacteria belonging to the genus *Xanthomonas* are predominantly plant pathogens. The exceptions are the non-pathogenic members of the genus that have been found in epiphytic as well as endoparasitic association with plants (Ercolani, 1978; Maas *et al.*, 1985; Mulrean and Schroth, 1982). The pathogenic xanthomonads are known to be responsible for a variety of bacterioses, including necrosis, gummosis, vascular and/or parenchymatous diseases on leaves (Bradbury, 1984; Vauterin *et al.*, 1991; van den Mooter and Swings, 1990).

Xanthomonas has a global distribution with a host range encompassing approximately 124 monocotyledonous and 268 dicotyledonous plant species. Included within this group of susceptible plants are a number of diverse, agriculturally and economically important crops (Leyns *et al.*, 1984). Listed amongst these are the crucifers, plants belonging to the family *Brassicaceae.* Members of this family include *Brassica oleracea* var. *capitata* (cabbage), *B. o.* var. *gemmifera* (Brussels sprouts), *B. o.* var. *botrytis* (cauliflower) and *B. o.* var. *italica* (broccoli). Bacterial diseases of these crops attributed to *Xanthomonas* infections are caused by various pathovars of *X. campestris* (Table 1.1). By far the most destructive disease of crucifers is black rot, which is caused by *X. campestris* pv. *campestris* (Williams, 1980; Bradbury, 1986).

Table 1.1: Diseases of crucifers caused by *Xanthomonas* and their geographical distribution (Swings and Civerolo, 1993)

Causal Organism	Disease	Geographical Distribution
X. *campestris* pv *aberrans*	leafspot of cauliflower	South Africa, Germany
X. c. pv. *armoraciae*	localised leafspot	USA, India, Turkey
X. c. pv. *campestris*	black rot	Global
X. c. pv. *raphani*	leafspot	USA, Brazil

1.3. General Characteristics of Bacteriophages

Although the action of bacteriophages in bacterial cultures must have been noted by numerous early biologists, these organisms were first described in 1915 by F. W. Twort, who isolated a filterable agent capable of changing the colonial morphology of staphylococci. However, interest in phages only followed the 1917 publication of a series of research papers by Felix d' Herelle, who coined the term bacteriophage (reviewed by Adams, 1959). Bacteriophages are now universally recognised as bacteria-specific viruses of diverse character.

1.3.1. Morphology and Chemical Composition

Bacteriophage particles are metabolically inert macromolecules consisting of a nucleic acid core surrounded by a protective protein coat or capsid (Wilson and Miles, 1975). The particles may be elongated with helical symmetry, spherical with icosahedral symmetry or a combination of icosahedral (the head) and helical (the tail) symmetry known as binal symmetry (Prescott *et al.*, 1990).

Bacterial viruses show great variation in both size and structural complexity. Capsid diameters range from 25-27 nm for members of the icosahedral family *Microviridae*, to the much larger head sizes of 65-80 nm for the T4 (*Myoviridae*) tailed phages. On the morphological level, phages can be differentiated by the presence or absence of a tail, which may be classified as contractile (*Myoviridae*) or non-contractile (*Siphoviridae, Podoviridae*); surface projections extending outwards from the vertices of the phage structure (*Corticoviridae, Tectiviridae, Microviridae*); a membranous outer envelope structure which comprises a lipid bilayer (*Lipothrixviridae, Fuselloviridae, Cystoviridae*). In one bacteriophage family (*Corticoviridae*), the lipid bilayer is enclosed between an inner and an outer protein shell. The lipid composition of these phages differs from that of the host, with 90% being phospholipids such as phosphatidylglycerol and phosphatidylethanolmelamine (Murphy *et al.*, 1995).

Based on these distinctive morphological characteristics, phages can be divided into 21 different morphotypes. In a comprehensive survey, including phages characterised since the first use of negative staining electron microscopy, it was apparent that morphotypes F3, G1 and G2 might be heterogeneous with respect to their nucleic acid content, as this characteristic was not always studied (Ackermann, 1996). Morphotype F4 is represented by a single archebacterial phage, SIRV (Zillig *et al.*, 1994), that closely resembles tobacco mosaic virus in

shape and which may represent a completely new phage family. Morphotype D2 was created for poorly known polyhedral, DNA-containing phages, which have yet to be classified. This morphological data, combined with the nature of the phage genome have permitted the classification of the 21 morphotypes into 12 taxonomic families (Table 1.2).

Despite the great variations observed in size and structural complexity, one of the most fundamental differences between phages and living bacterial cells lies in their nucleic acid content. Bacteria contain both DNA and RNA, whereas phages have been known to harbour only one type of nucleic acid - either DNA or RNA - which may be double or single stranded, and in the case of DNA, a linear or closed circular molecule.

Recently, however, Witte *et al.* (1997) isolated the bacteriophage, ϕCh1, from the halophilic archeon, *Natronobacterium magadii.* This phage morphologically resembles members of the family *Myoviridae*, but differs by harbouring a double-stranded linear DNA genome as well as several RNA species. It appears that most of the RNA species are host-encoded, although weak signals were obtained when phage DNA hybridised with the RNA packaged into the mature phage particles. The reason for the presence of the RNA as well as its function is as yet unknown. In contrast, the *Bacillus subtilis* phage, ϕ29, is known to encode a small RNA transcript required for *in vitro* packaging. However, this RNA is associated with a partially completed head precursor and not the mature phage particle (Bailey *et al.,* 1990, Guo *et al.,* 1987a & b).

Table 1.2: Classification of Bacteriophages

Morphotype	Family	Genus	Type species	Morphology	Enveloped	Nucleic Acid
A1-A3	*Myoviridae*	T4-like phages	coliphage T4	I, CT	NO	ds DNA, linear
		P1-like	P1	I, CT	NO	ds DNA, linear
		P2-like	P2	I, CT	NO	ds DNA, linear
		Mu-like	Mu	I, CT	NO	ds DNA, linear
		SPO1-like	SPO1	I, CT	NO	ds DNA, linear
		phiH-like	phiH	I, CT	NO	ds DNA, linear
B1-B3	*Siphoviridae*	λ-like phages	coliphage λ	I, NT	NO	ds DNA, linear
		psiM-like	psiM	I, NT	NO	ds DNA, linear
		T5-like	T5	I, NT	NO	ds DNA, linear
		T1-like	T1	I, NT	NO	ds DNA, linear
		c2-like	c2	I, NT	NO	ds DNA, linear
		L5-like	L5	I, NT	NO	ds DNA, linear
D3	*Corticoviridae*	*Corticovirus*	Alteromonas phage PM2	I, apical spikes	NO	ds DNA, circular
C1-C3	*Podoviridae*	"T7-like phages"	coliphage T7	I, short NT	NO	ds DNA, linear
		ϕ29-like	ϕ29	I, short NT	NO	ds DNA, linear
		P22-like	P22	I, short NT	NO	ds DNA, linear

Table 1.2 continued

D4	*Tectiviridae*	*Tectivirus*	Enterobacteria phage PRD1	I, apical spikes	NO	ds DNA, linear
F3	*Lipothrixviridae*	*Lipothrixvirus*	*Thermoproteus* virus 1	F	YES	ds DNA, linear
G2	*Fuselloviridae*	*Fusellovirus*	*Sulfolobus* virus 1	ovoid	YES	ds DNA, circular
G2	*Plasmaviridae*	*Plasmavirus*	*Acholeplasma* phage L2	pleomorphic	YES	ds DNA, circular
F1-F2	*Inoviridae*	*Inovirus*	coliphage fd	F	NO	ss DNA, circular
D1	*Microviridae*	*Microvirus*	coliphage ϕX174	I, apical spikes	NO	ss DNA, circular
		Bdellomicrovirus	*Bdellovibrio* phage MAC1	I	NO	ss DNA, circular
		Spiromicrovirus	*Spiroplasma* phage 4	I	NO	ss DNA, circular
		Chlamydiamicrovirus	*Chlamydia* phage 1	I	NO	ss DNA, circular
E2	*Cystoviridae*	*Cystovirus*	*Pseudomonas* phage ϕ6	I	YES	ds RNA, 3 linear
E1	*Leviviridae*	*Levivirus*	enterobacteria phage MS2	I	NO	ss RNA, +strand linear
		Allolevivirus	enterobacteria phage Qβ	I	NO	ss RNA, +strand linear

I = icosahedral; F = filamentous; CT = contractile tail; NT = non-contractile tail

1.3.2. Phage Life Cycles

The production of progeny phage particles requires the synthesis of multiple copies of the phage genome and structural proteins. The free phage is a metabolically inert particle whose genome, upon injection into a suitable host, is able to direct the synthesis of progeny phage using some of the host cell's machinery in the process (Wilson and Miles, 1975). Upon infecting a bacterium, there are two major pathways of phage replication that can occur: the lytic or the lysogenic pathway (Luria and Darnell, 1967).

1.3.2.1. The Lytic Cycle

Phages that engage in the lytic cycle and ultimately lead to lysis of the bacterial host, are said to be virulent. Four stages of phage infection during the lytic cycle have been defined: (i) adsorption to the host, (ii) nucleic acid injection or penetration, (iii) intracellular multiplication and maturation, and (iv) bacterial lysis accompanied by the release of newly formed viral progeny.

During the first stage of infection, the phages recognise and attach to specific receptor sites on the bacterial cell surface. Cell wall lipopolysaccharides/lipo-oligosaccharides, outer membrane proteins, flagella or pili may serve as phage receptor sites. The peptidoglycan layer as well as teichoic acids have also been shown to function as primary receptors for phage receptor-binding proteins (Wendlinger *et al.*, 1996). As a rule, a phage can only recognise one receptor protein. However, a number of phages exhibit dual receptor specificities. For example, bacteriophage T4 has the ability to recognise two chemically different receptors: diglucosyl residues in the lipopolysaccharides as well as the outer membrane protein OmpC of *Escherichia coli* (Wilson *et al.*, 1970; Yu and Mizushima, 1982). Other outer membrane proteins that function as phage receptor sites include FadL, a fatty acid specific transport protein which serves as the major receptor for phage T2 (Black, 1990); the well characterised malto-dextrin specific pore, LamB, recognised by phage λ (Gehreng *et al.*, 1987); and the structural protein OmpA (Morona *et al.*, 1985).

The exact details of the process of nucleic acid ejection from the phage are not completely understood. However, extensive studies involving the phages T4 and T7 have provided evidence for the requirement of ATP and a membrane potential for the transport of the phage DNA through the bacterial inner membrane and into the cytoplasm (Kalasauskaite *et al.*,

1983; Zimkus *et al.*1986, cited by Karam, 1994). Upon entry of the nucleic acid into the cytoplasm, a series of events usually mediate shutoff of all or most host macromolecular synthesis, and the host cell components are directed towards synthesising phage components. The mechanism of replication and transcription of the nucleic acid, of course, depends upon the nature of the phage genome. Following phage morphogenesis and nucleic acid packaging is the final step of the lytic cycle: lysis, a process mediated by phage encoded products (Abedon, 1994; Campbell, 1994).

1.3.2.2. The Lysogenic Cycle

Bacteriophages that establish lysogenic relationships with their bacterial hosts are said to be temperate phages. Because of the stable hereditary nature of lysogeny, its recognition usually depends upon the chance isolation of a sensitive, indicator bacterial strain which will be lysed by the phage, resulting in plaque formation on solid media; or until the lytic cycle is induced by prevailing environmental conditions or factors such as UV radiation and chemical mutagens. After entering the bacterial cell, the temperate phage genome persists within the host in a non-infectious form and is reproduced at the same time as the bacterial genome to generate a clone of infected cells that may grow and divide for many generations while appearing perfectly normal (Hayes, 1964). Occasionally the phage genome enters the lytic cycle of development to produce progeny which are subsequently released by bacterial lysis.

Often mistaken for lysogeny is the phage carrier-state known as pseudolysogeny. Pseudolysogenic relationships, as reviewed by Lwoff (1953), are characterised by heterogeneous populations consisting of bacteria rapidly alternating between sensitive and resistant phenotypes in a more or less stable equilibrium with phage production. Maintenance of the phage is thought to be due to infection of sensitive cells in the population. In *Bacteroides fragilis* and *Shigella dysenteriae* populations, the carrier state has been ascribed to the production of a thick capsule which interferes with phage production and the loss of phage receptors, respectively (Booth *et al.*, 1979; Li *et al.*, 1961)

The temperate phages have a wide variety of lysogenisation modes. The phage genome may exist persistently in the host as an episome, as in the case of phage P1 and filamentous phages. The P1 DNA genome replicates in synchrony with the host chromosome, resulting in a copy number equal to the host chromosome (Prentki *et. al.*, 1977). In contrast, the

single-stranded DNA genome of filamentous phages (*e.g.* members of the family *Inoviridae*, such as M13) replicates independently of the host chromosome via a double-stranded replicative intermediate (Ray, 1978). However, infection by filamentous phages leads neither to lysis nor lysogeny. Instead, mature phage particles are continuously extruded through the cell wall while the infected bacteria continue to grow and reproduce (Jacob and Wollman, 1959; Hayes, 1964). This is unlike true lysogeny in which the phage genome retains the ability to produce progeny, but remains latent until specific conditions cause the induction of the lytic cycle.

Another mode of lysogeny involves the phage genome persisting in the bacterium by integrating into the host chromosome, and reproducing in synchrony with the bacterial genome. Most temperate phages resemble bacteriophage λ whose double-stranded DNA genome integrates into a highly specific chromosomal site with the aid of a phage-encoded, site-specific integrase (Weisberg and Landry, 1983). However, for some phages, the integration may be completely random, as in the case of bacteriophage Mu which combines the properties of a temperate phage and a transposable element (Taylor, 1963). Chromosomal integration has long been thought to be restricted to double-stranded DNA phages (Barksdale and Arden, 1974; Freifelder, 1983). Recent reports in literature, however, confirm that this phenomenon is not confined to double-stranded DNA-containing phages with icosahedral symmetry, as was first thought. The filamentous, single-stranded, DNA-containing phages VSK from *Vibrio cholera* 0139, ϕXo from *X. oryzae* pv. *oryzae*, ϕXv from *X. campestris* pv. *vesicatoria*, Cflt and Cf16, both of which infect *Xanthomonas campestris* pv. *citri,* were shown to integrate the double-stranded replicative form (RF) of their genomes into the chromosome of their respective hosts (Kar *et al.*, 1996; Lin *et al.*, 1994; Kuo *et al.*, 1987(a&b); Dai *et al.*, 1987).

Dai *et al.* (1987) proposed the term "neolysogenization" to delineate this unique form of lysogenisation, as it comprises a combination of characteristics of well known temperate phages and filamentous phages. In the neolysogenization process, the initial release of large numbers of phage particles - reminiscent of filamentous phages - is followed by a decrease as the infectious cycle progresses. Finally the phage genome integrates into the host chromosome. Neolysogenization differs from the lysogenization of temperate phages in the fact that the free RF DNA coexists with the integrated form and continues to replicate

independently. Although cells exhibit a tendency to eventually eliminate the free RF and retain only the integrated form, this does not always happen.

Stewart and Levin (1984) put forward two major hypotheses to explain the advantages of lysogeny. The first is that lysogeny confers immunity to superinfection by the same phage strain. This means that the bacterial hosts have a competitive advantage over nonlysogenised cells that can be infected and lysed. The second explanation is that lysogeny provides a survival strategy to maintain phage populations when the availability of potential hosts is too low for populations to be maintained by lytic infection. Also, in adverse conditions such as nutrient limitation, it is advantageous for the phage to enter the lysogenic phase of infection and become dormant at the same time as its host. The reason for this is that the degradation - by bacteria - of their own mRNA and protein prior to dormancy, would result in a permanent interruption of the phage reproductive cycle (Prescott *et al.*, 1990).

1.3.3. Host Specificity

Phages are associated with most bacterial families and can often be isolated from wherever a particular bacterial species occurs in nature. As is true of plant and animal viruses, phages vary in their host specificity. Some phages are highly specific, infecting only a particular pathovar, while other phages have a wider host range, being specific only at the species level or even the generic level (Adams, 1959). Phages have also been known to infect genera of different families, *e.g.* a phage of *Pasteurella pestis* (Family *Pasteurellaceae*) is able to infect members of the genera *Salmonella* and *Shigella*, both of which belong to the family *Enterobacteriaceae* (Lazarus and Gunnison, 1947); and phage T4 which infects *E. coli* and *Shigella* has a rare host range mutant that is capable of infecting the evolutionarily distant *Yersinia pseudotuberculosis* I (Tétart *et al.*, 1996).

The host range is not an invariant characteristic of a particular phage, however, because the host range can be altered as a result of mutation or as a consequence of phenotypic modification. Whereas a mutation is permanent and heritable, a phenotypic modification of phage properties implies a non-hereditary change, usually attributable to specific growth conditions (reviewed by Adams, 1959). Phenotypic alterations are therefore reversible in the immediate descendants of the phage in the absence of the causal factor(s).

Bacteriophages recognise potential hosts through highly specific binding to bacterial receptors located on the cell surface. Changes in the surface of a bacterium, as a result of mutations and antigenic changes, may affect the host range by blocking the adsorption of a phage otherwise able to reproduce in such a host.

When under such selective pressure, phages employ an array of different methods whereby they can extend or alter their host ranges:
(i) possession of invertible DNA segments which allow the phage to alternate between two sets of host ranges,
(ii) point mutations in tail fibre genes,
(iii) acquisition of a segment of a tail fibre gene from a different phage, i.e. horizontal gene transfer,
(iv) picking up a gene fragment that has been left behind by another phage after imprecise excision from the bacterial chromosome.

Accumulation of molecular data concerning receptor recognition has provided some insight into the underlying molecular mechanisms, accompanied by the further understanding of some of the factors which might lead to altered host ranges of phages. Examples of extended host ranges are seen in the Ox2 (a T-even phage) host range mutants. A mutation in the mutant, Ox2h10, resulted in the ability to utilise OmpC and OmpX in addition to OmpA as receptors, and in the mutant, Ox2h12h1.11, it resulted in the phage being able to bind to lipopolysaccharide (Drexler *et al.* 1991).

The inversion of the G region of phage Mu, which is involved in tail fibre production and assembly, was found to result in phages which adsorb on different bacterial strains from phages with the G region in the opposite orientation (reviewed by Koch *et al.*, 1987 and Toussaint, 1985). This region shares sequence homology with regions containing the genes encoding the tail fibre structural and assembly proteins of the T-even phages (around genes 37 and 38), P1 (the invertible C-region), P2 (the gene H region) and λ (ORF 314 and 401) (reviewed by Karam, 1994; Haggård -Ljungquist *et al.*, 1992; Montag and Henning, 1987). Apart from Mu and P1, there is no evidence that an inversion event plays a role in determining the host ranges of the other phages. According to Haggård-Ljungquist *et al.* (1996), the sequence similarity displayed by these phages may be due to horizontal gene

transfer during the course of phage evolution. The detection of sequences homologous to genes 36 and 37 of the T-even phage, T2, on the *E. coli* chromosome by Riede *et al.* (1985), does not rule out the possibility that the phages may have acquired segments of their tail fibre genes by picking up the sequences from the host chromosome.

1.4. The Importance of Bacteriophages

1.4.1. Historical Overview

In the decades subsequent to the discovery of bacterial viruses, these relatively simple organisms have made considerable contributions towards the understanding of viruses in general, and to molecular genetics and biotechnology. The reason phages are regarded as the ideal material for genetic studies is because of the ease with which mutants can be isolated, genetic crosses made, and the numerous progeny particles analysed.

Some of the most noteworthy milestones in the history of phage research include the realisation of the triplet nature of the genetic code from work done with T4 (Crick *et al.*, 1961); proof of the existence of mRNA and its function as the template for protein synthesis, which was elucidated in studies of cells infected with T4 (Brenner *et al.*, 1961); the demonstration of the co-linearity of gene and protein structure in a study of the *trp* region of the *E. coli* chromosome using the generalised transducing phage, P1 (Yanofsky *et al.*, 1964).

Compared to these monumental contributions to molecular genetics, the use of specific bacteriophages to detect the presence of a particular bacterial strain in a mixture of organisms as well as in phage typing schemes may seem just a little mundane. However the limited host range of many phages makes them useful for distinguishing between bacteria that are shown to be identical by serological and other tests, as well as identifying an organism as a member of a particular species or larger taxonomic group.

1.4.2. Phage Genetics

Not only can bacterial genes be transferred by conjugation and transformation, but they can also be transferred between bacteria by phages in a process known as transduction. There are two fundamentally different transduction mechanisms:

(i) generalised transduction, which was discovered by Zinder and Lederberg (1952) and demonstrated for phage P22 of *Salmonella typhimurium.* This occurs during viral assembly when random fragments of the bacterial genome may be packaged along with phage DNA. Generalised transduction has been used to study the linkage of two or more genes on the bacterial chromosome: the greater the frequency of co-transduction, the closer the genes are to each other.

(ii) Specialised transduction, first demonstrated for phage λ (reviewed by Reanney and Ackerman, 1982) results from the imprecise excision of the phage genome from the host chromosome. In this mechanism, bacterial DNA adjacent to the phage attachment (*att)* sites are excised along with the prophage. This allows for the purification and identification of bacterial genes close to the phage *att* sites.

Efficient mutagenesis and thus the genetic manipulation of bacteria has been achieved by using temperate phages such as Mu (Taylor, 1963; Bukhari and Zipzer, 1972) - which insert randomly into the bacterial chromosome, occasionally resulting in the inactivation of bacterial genes - and the P4::Tn5 suicide transposon vectors (Polissi *et al.*, 1992), which exploit the broad host range of the satellite phage P4.

1.4.3. Industrial/Economic Importance

In any food-related industry, bacterial contaminants are a serious problem. Bearing this in mind, Loessner *et al.* (1997) developed an effective method of using phages in bacterial detection, which decreased the time required for the detection process as well as allowing very low levels of bacterial contamination to be detected. Bennet *et al.* (1997) took this one step further by employing a biosorbant - which consists of phage immobilised to a solid phase - that effectively removes the contaminating bacteria from mixed populations. These methods could be applied to a variety of target organisms and thus form reliable detection and separation systems in the field of food microbiology.

In molecular biology, phages remain important as cloning vectors (*e.g.* M13, λ, cosmids), sources of enzymes (T4 DNA ligase) and surface expression systems (*e.g.* M13) (Bolivar *et al.*, 1977; Hohn and Collins, 1980; Onda *et al.*, 1995).

When characterising pathogens, the major objective is to determine the basis of the organism's ability to cause disease. Temperate phages have often been found to carry genes which confer new properties on their hosts. It is now known that a variety of toxin and virulence genes expressed by bacteria are in fact bacteriophage-encoded (Table 1.3). This phage-mediated acquisition of new traits is called lysogenic conversion. The fact that phage genomes code for toxins and other virulence factors which serve no direct purpose for the phage itself, indicates that there must be some kind of evolutionary advantage in maintaining these genes. Cheetham and Katz (1995) suggest that the advantage to the phage may be the enhanced replication of the bacteria which have undergone lysogenic conversion.

Table 1.3: Bacteriophage-encoded virulence determinants

Phage	Host	Phage Product	Reference
ϕCTX	*Pseudomonas aeruginosa*	cytotoxin	Hayashi *et al.*, (1993)
ϕ42, ϕA1, ϕA3	*Staphylococcus aureus*	staphylokinase, enterotoxinA	Coleman *et al.*, (1989)
CTXϕ	*Vibrio cholera*	cholera toxin	Waldor and Mekalanos (1996)
β	*Corynebacterium diptheriae*	diptheria toxin	Barksdale and Arden (1974)
933J	*Escherichia coli*	shiga-like toxin	Newland *et al.*, (1985)

1.5. Phages of *Xanthomonas*

The phages of *Xanthomonas* have been well documented, with a number of species of *Xanthomonas* and pathovars of *X. campestris* found to be infected with phages (Table 1.4). The phages associated with *Xanthomonas* are mainly filamentous or tadpole-shaped, with a polyhedral head and a tail. They can be divided into four morphological groups, *viz.* filamentous phages, phages with polyhedral heads which possess contractile tail sheaths or non-contractile flexous tails, or short wedge-shaped tails (Table1.4).

The phages described for the genus harbour either double or single-stranded DNA as their genetic material. The phage Xp12 which infects *X. oryzae,* the pathogen of bacterial leaf blight of rice, is distinguished from other *Xanthomonas* phages by the presence of an unusual pyrimidine base in its DNA. The pyrimidine base, cytosine, is completely replaced by 5-methylcytosine (Kuo *et al.*, 1968). Unusual base replacement has been reported to occur in other bacteriophages, *e.g.* the T-even phages of *E. coli* and phage G of *Bacillus megatherium* have the cytosine of their DNA replaced by glucosylated 5-hydroxymethylcytosine (Wyatt, 1953; Donelli *et al.*, 1975) and the bacteriophage SP8 of *Bacillus subtilis* contains 5-hydroxymethyluracil instead of thymine (Kallen *et al.*, 1962). Such base replacements may serve to protect the phage DNA from host encoded restriction enzymes as well as assisting in distinguishing phage DNA from host DNA, thereby preventing the accidental breakdown of phage DNA.

Both temperate and virulent phages have been found to infect *Xanthomonas,* with the latter phages predominantly having polyhedral heads and tails (Table 1.4). Studies of single-stranded DNA phages Cflt, Cf16, ϕXo and ϕXv have led to a greater insight into the complexity of phage survival strategies within their hosts as well as the coining of the term, neolysogenization, as a result of their ability to lysogenise their host by integrating into the chromosome.

Host range studies have demonstrated that several phages active against *Xanthomonas* have a high degree of specificity at the pathovar level, such as *X. campestris* pv. *campestris* (Liew and Alvarez, 1981b; Tseng, 1990), *X. oryzae* N5850 (Kamiunten and Wakimoto, 1979), *X. oryzae* pv. *oryzae* and *X. campestris* pv. *vesicatoria* (Lin *et al.*, 1994), *X. campestrix* pv. *pruni* (Du Plessis *et al.*, 1981) and *X. campestris* pv. *citri* (Dai *et al.*, 1980). However, other *Xanthomonas* bacteriophages such as the SP phages isolated from *X. o.* pv. *oryzicola* (Goto, 1965), as well as phages isolated from *X. campestris* pv. *holicola, X. campestris* pv. *vesicatoria* and *X. campestris* pv. *pruni* (Alippi, 1989), are only specific at the generic level and exhibit a low degree of specificity at the pathovar level.

1.6. Project Aims

The ultimate goal of the project was to characterise the putative phage of the chocolate spot pathogen with respect to its physicochemical, serological and biological properties. The

project was prompted not only by the particle morphology combined with the absence of obvious host cell lysis, but also the idea that like certain *Xanthomonas* phages, this putative phage might be helpful in the detection of the chocolate spot pathogen. However, the mucoid quality of the "chocolate spot" cultures created difficulties in the purification of the particles. Thus, the initial aim was to develop a purification protocol that would yield a sufficiently high quality and quantity of the particles which could then be used for the characterisation process. Another aspect of this study, using electron microscopy and serological techniques, was to determine whether the presence of the phage could serve as a reliable means for detection of the chocolate spot pathogen. Furthermore, cloning and sequencing of parts of an extrachromosomal DNA element was undertaken in order to gain insight into possible phage genes.

Table 1.4. The characteristics of phages infecting *Xanthomonas* species

Bacterial Host	Phage	Morphology[a]	Biology	Reference
X. oryzae	XP12	H, NT	virulent	Kuo, 1968
X. oryzae strain N5845	Xf2	F	temperate	Kamiunten & Wakimoto, 1979
X. oryzae strain 507	Xf	F	temperate	Kuo *et al.*, 1969
X. oryzae pv. *oryzae*	ϕXo	F	temperate	Lin *et al.*, 1994
X. campestris	HP^1	O, CT	virulent	Liew & Alvarez, 1981a
X. campestris	HP^3	H, CT	virulent	Liew & Alvarez, 1981a
X. campestris	HT^7	H, CT	virulent	Liew & Alvarez, 1981a
X. campestris	HT_{3b}	H, CT	virulent	Liew & Alvarez, 1981a
X. campestris	A342	H, NT	virulent	Liew & Alvarez, 1981a
X. campestris	HXX	H, NT	virulent	Liew & Alvarez, 1981a
X. campestris	R R68	H, ST	virulent	Liew & Alvarez, 1981a
X. c. pv. *campestris*	ϕL7	H, NT	virulent	Su *et al.*, 1990
X. c. pv. *campestris*	ϕLf	F	temperate	Tseng *et al.*, 1990
X. c. pv. *campestris*	XTP1	I, CT	virulent	Weiss *et al.*, 1994
X. campestris pv. *citri*	Cf	F	temperate	Dai *et al.*, 1980
X. campestris pv. *citri*	Cf16	F	temperate	Lin *et al.*, 1994
X. campestris pv. *citri*	Cflt	F	temperate	Kuo *et al.*, 1987a
X. c. pv. *cucurbitae*	XCU-P1	P+T	virulent	Alippi, 1989
X. c. pv. *cucurbitae*	XCU-P3	P+T	virulent	Alippi, 1989
X. c. pv. *holicola*	XHOL-P1	P+T	virulent	Alippi, 1989
X. c. pv. *mangiferaeindicae*	Xm1	I, NT	virulent	White *et al.*, 1985
X. c. pv. *mangiferaeindicae*	Xm2	F	virulent	White *et al.*, 1985
X. c. pv. *mangiferaeindicae*	Xm3	I, CT	virulent	White *et al.*, 1985
X. c. pv. *mangiferaeindicae*	Xm4	I, CT	virulent	White *et al.*, 1985
X. c. pv. *vesicatoria*	φXv	F	temperate	Lin *et al*, 1994

[a] F, filamentous; I, icosahedral head; O, octagonal head; H, hexagonal head; CT, contractile tail; NT, noncontractile tail; ST, short wedge-shaped tail; P, polyhedral; T, tailed

CHAPTER 2

The Purification and Physicochemical Characterisation of the bacteriophage

Contents

The purification and physicochemical characterisation of the bacteriophage

Summary

The optimal bacterial growth conditions for production of bacteriophage-like particles by the causal agent of chocolate spot disease of crucifers was established and a purification protocol developed. The particles are isometric with a diameter of 33.5±4.5 nm, a sedimentation coefficient of 85S and a density of 1.347 g/ml in caesium chloride. The particles are thought to be a novel *Xanthomonas* bacteriophage since immunoelectroblot analysis showed that they were not outer membrane vesicles of bacterial origin. The particles contain a single-stranded DNA genome and 5 proteins of molecular weights 94, 40, 32, 25.7 and 21.8 kDa. The ^{32}P-labelled genome hybridised to a 53.9 kb extrachromosomal DNA element which is too large to be packaged into a capsid of 33.45 nm in diameter. The phage is a relatively stable particle with respect to pH, solvents and at temperatures of 40°C and 60°C.

2.1. Introduction

Because of the large number of viruses that have already been studied and placed into taxonomic groups on the basis of their morphological, physical, biophysical and biological characteristics, it is necessary in most cases to measure only a few characteristics such as the host range, morphology, size, nucleic acid type and antigenicity of a new virus in order to place it into the correct taxon. Exceptions occur when a new virus does not have a familiar set of properties and in such cases, comprehensive characterisation of all virion properties is required (Murphy *et al.*, 1995).

Initial electron microscopic observations of the phage-like particles associated with PCB 22 (Figure 1.1.), an isolate of the chocolate spot pathogen, showed their morphology to be unlike that of *Xanthomonas* phages reported in literature. Before characterisation could proceed, it was deemed necessary to establish that these particles were indeed phages and not vesicles ("blebs") of bacterial origin. This was even more necessary because of the absence of plaque formation and the fact that several Gram negative bacteria, including *X. campestris* pv. *oryzae*, have been reported to produce non-viral vesicles (Grenier and Mayrand, 1987; Poirier *et al.* 1979; Nowotny *et al.* 1982; Kamiunten and Wakimoto, 1981). These vesicles are mostly spherical in shape and might easily be mistaken for phages. The vesicles are the result of budding of the outer membrane, and evidence suggests that vesiculation may occur in response to environmental stress (Koga *et al.*, 1985; Thompson *et al.*, 1985). In the case of *X. campestris* pv. *oryzae*, vesiculation occurs in response to infection by the filamentous phages Xf2 and Xf, and is accompanied by severe destabilisation of the cell wall (Kamiunten and Wakimoto, 1981; Kamiunten and Wakimoto, 1982).

A reliable purification protocol was required to allow characterisation in order to help establish the authenticity or otherwise of the putative phage. The isolation and purification of a phage essentially involves the following procedures.

(i) Selection of a suitable growth medium for the host cells. The choice of medium is important as the growth and metabolism of the bacteria, and ultimately the yield of the phage, depends upon the nutritional environment of the host (Adams, 1959).

(ii) Extraction of the phage present in the infected host cells and clarification of the phage extract.

(iii) Concentration of the phage and purification by removal of low molecular weight contaminants.
(iv) Further purification by rate zonal or isopycnic density gradient centrifugation.

During the purification of a virus, particularly a previously uncharacterised one, it is essential to assess the relative efficiency of each step in the purification procedure. This is necessary first, to determine the amount of loss of the virus in each step; second, to monitor the phase into which the virus is separated; and third, to assess the efficiency with which contaminants are removed. Ideally, a pure, homogeneous virus preparation would contain a population of identical particles completely free of impurities. However, in practice, this can never be achieved as any virus preparation may be morphologically homogeneous, but biologically heterogeneous, *e.g.* a preparation consisting of a mixture of infective and non-infective particles, or of wild-type and mutant virus strains (Francki, 1972).

This chapter reports the isolation and development of a purification protocol for the putative phage. It further reports the determination of the morphological and physicochemical properties of the particles.

2.2. Materials and Methods

2.2.1. The bacterial isolates and growth conditions

PCB 22, isolated from the leaves of infected cabbage seedlings and determined to be a novel strain variant of *Xanthomonas campestris* pv. c*ampestris* (Kariem, 1994), was the first isolate found to be associated with the putative phage (Chapter 1), and was thus used as the host for the propagation, isolation and purification of these particles. Long term storage of PCB 22 and *Xanthomonas campestris* pv. *vasculorum* NCPPB 5757, used in an immunoassay, was carried out at -20°C in 20% glycerol. Working cultures were subcultured and maintained on nutrient agar (Biolab) and grown at 30°C.

2.2.2. Bacteriophage propagation and purification

Several preliminary attempts at isolating the putative bacteriophage in sufficient quantities, and purifying it to produce a sample of acceptable quality for further analysis, were unsuccessful due to the presence of a mucoid substance. Changing the growth medium from

solid to liquid, varying the composition of the media [standard mineral base (SMB) (Appendix A.1), SMB with 0.01%-0.5% yeast extract] and eliminating the mucoid substance by digestion with α-amylase was attempted. The viscosity of the phage-containing preparation also affected the efficiency of purification by sucrose gradient centrifugation. Due to this fact, the efficacy of different percentage sucrose gradients, *viz.* 10-40%, 15% layered onto 60% and 5-20% (w/v), as well as the length of time required for rate zonal density gradient centrifugation (2.5-8 hours) was investigated. The buffers TEN (Tris, EDTA, NaCl), phosphate buffered tryptone (PBT) and phosphate buffered saline (PBS) were evaluated for phage extraction (Appendix A). The effect of the addition of $MgSO_4$ to PBS was also investigated.

Attempts were made to increase the final yield of the phage through concentration with polyethylene glycol (Mr 6000), disruption of the bacterial cells by treatment with chloroform or French pressing, as well as a combination of lysozyme treatment and sonication.

The following method proved to be the most effective for the isolation and purification of the particles from PCB 22. For each isolation, PCB 22 was grown on 2-3 L of nutrient agar (Biolab) and incubated at 30°C for 60-72 hours. The particles were harvested from nutrient agar plates by the addition of 5 ml PBS pH 7.2 containing 5 mM $MgSO_4$ to each of the plates, which were then left at room temperature for a minimum of one hour with occasional shaking. The suspensions were collected and the cell debris removed by low speed centrifugation (10 000 rpm for 10 min at 4°C in a Beckman JA14 rotor). The supernatant was concentrated by ultracentrifugation in a Beckman 50 Ti or 50.2 Ti rotor (40 000 rpm for 2 hours at 4°C). The pellets were resuspended in PBS-$MgSO_4$ pH 7.2 and subjected to a final low speed centrifugation step to remove any insoluble components. The phage was further purified by rate zonal density gradient centrifugation in 5-20% (w/v) sucrose gradients at 25 000 rpm for 2.5 hours (Appendix B.1.). The sucrose was removed by dialysis against PBS-$MgSO_4$ pH 7.2, or by diluting the suspension 1:4 with PBS-$MgSO_4$ pH 7.2, followed by concentration by ultracentrifugation in a Beckman 50 Ti rotor (40 000 rpm for 1.5 hours at 4°C). The rate zonal centrifugation and final ultracentrifugation steps were repeated twice in order to obtain a relatively homogeneous suspension of particles. The pellets were resuspended in PBS-$MgSO_4$ pH 7.2 and stored at 4°C.

2.2.3. Immunological test

2.2.3.1. Antiserum preparation

Following the purification procedure described in section 2.2.2., a suspension containing 0.8 µg/ml of protein (determined using Bradford protein assays; Appendix B.3.1) was made up to a volume of 1 ml with PBS, before being mixed with Freund's incomplete adjuvant (1:1 ratio). This sample was injected into a rabbit with booster injections of the antigen being administered every 7 days just before the rabbit was bled.

2.2.3.2. Immunoelectroblotting

Immunoelectroblotting (IEB) assays were used to eliminate the possibility that the particles observed by electron microscopy might be vesicles of bacterial origin rather than phages. Since the infected host, PCB 22, could not be used in this experiment, *X. campestris* pv. *vasculorum*, one of only two *Xanthomonas* species available at the time which was not associated with phage-like particles, was used as the source of bacterial proteins. The sucrose gradient-purified preparations (Section 2.2.2.) and bacterial samples were disrupted in an equal volume of protein disruption buffer (Appendix B.3.2). The samples were electrophoresed through 12% acrylamide gels using the discontinuous buffer system of Laemmli (1970) (Appendix B.3.2). The proteins were electrophoretically transferred to nitrocellulose membrane (0.45 µm, MSI) and the antibody binding step, using the antiserum raised against the phage, was performed essentially according to the method of Towbin *et al.* (1979) (Appendix B.4.1).

2.2.4. Biophysical and physical characterisation

2.2.4.1. Morphology

To determine the morphology, purified preparations of the particles were adsorbed onto carbon coated grids. The particles were negatively stained with 2% (w/v) uranyl acetate pH 4.2 (Griffin, 1990) and viewed in a ZEISS EM109 transmission electron microscope (Appendix B. 5.1). The size of the phage was calculated by measuring the diameters of the particles viewed on the photographic negative using a Mitutoyo toolmakers microscope.

2.2.4.2. Relative sedimentation coefficient

The approximate sedimentation coefficient of the particles was determined by rate zonal density gradient centrifugation (Appendix B.1) of a purified phage sample layered on a 10-40% sucrose gradient and centrifuged for 2.5 hours at 25 000 rpm in a Beckman SW 28 swinging bucket rotor. The plant viruses brome mosaic virus (BMV) and turnip yellow mosaic virus (TYMV) were used as internal standards. The high sedimentation coefficients of λ and T4 resulted in the rapid sedimentation of these bacteriophages, precluding their use in this experiment.

2.2.4.3. Buoyant density

The buoyant density of the particles in caesium chloride was determined by isopycnic density gradient centrifugation (Appendix B.2).

2.2.4.4. Protein analysis

The purified particles were disrupted in an equal volume of protein disruption buffer (Appendix B.3.2) at 100°C for 10min. The number and molecular weights of the proteins were determined by SDS-PAGE using the discontinuous system of Laemmli (1970) (Appendix B.3.2). The Pharmacia low molecular weight protein markers were used as standards and the gels were stained with Coomassie Brilliant Blue solution (Appendix B.3.3). The molecular weights of the proteins were calculated from the linear relationship between the distance of migration and the log of the molecular weight of the standard proteins. The stained gels were scanned with a GS 300 reflectance/absorbance (585nm) scanning densitometer (Hoefer Scientific Instruments). The relative proportion of each protein was estimated by calculating the percentage area beneath the peaks of the densitometer tracings.

2.2.4.5. Nucleic acid analysis

(a) Isolation and enzymatic digestion

To determine the nature and size of the putative phage genome, the nucleic acid was isolated from sucrose gradient-purified samples. Prior to nucleic acid isolation, particles were treated with 450 U of DNase io the presnce of 10 mM $MgSO_4$ and incubated at 37°C for 2 hours, in order to remove any contaminating bacterial DNA. The reaction was stopped by the addition of 0.5 M EDTA. Contaminating RNA was eliminated with the addition of 50 µg/ml RNase

A and incubation at 37°C for one hour. To isolate the nucleic acid, 2% SDS and 100 µg/ml proteinase K were added to the phage preparation before overnight incubation at 55°C, followed by extraction with phenol:chloroform:isoamyl alcohol (25:24:1). The form and type of nucleic acid were determined by staining both the purified particles and the isolated nucleic acid with acridine orange according to a modified method of Bradley's (1966) phage nucleic acid staining technique (Appendix B.8). The nucleic acid was tested for sensitivity to 444 ng/µl of RNase-free DNase I (Weiss *et al.*, 1994) and 30 µg of RNase A in 0.3 M NaCl by incubating the samples at 37°C for 1 hour (Williamson, 1988). Enzymatic digestion was monitored by agarose gel elctrophoresis (Appendix B.10).

(b) Isolation of extra-chromosomal DNA from PCB 22

Extra-chromosomal DNA was prepared from the bacterial precipitate obtained after the initial low speed centrifugation step of the particle purification procedure. The DNA was isolated using the method for large scale preparation of plasmid DNA and purified by caesium chloride-ethidium bromide density gradient centrifugation (Appendix B.7), which eliminates contamination by bacterial DNA. Using the nucleic acid isolated from the putative phage particles and labelled with ^{32}P by the random priming method as a probe, the Southern hybridisation technique (Appendix B.14.2) was used to determine whether this DNA was a possible replicative form of the putative phage genome or of plasmid origin.

(c) Molecular weight estimation

The size of the putative phage replicative form was estimated from the restriction sizes obtained after enzymatic digestion (Appendix B.9), using the restriction enzymes *Eco*RI, *Hin*dIII and *Sal*I.

2.2.4.6. pH stability

Equal quantities of purified particles were resuspended in the appropriate buffer. To ensure that the concentrations of each sample was equivalent, the amount of protein was determined using Bradford assays (Appendix B.3.1). For pH 7-10, 50 mM PBS was used, whereas 50 mM sodium acetate was used for pH 4-6. The suspensions were incubated at room temperature. Electron microscopic (EM) analysis was conducted to observe the effect of pH on the particle morphology. Changes in antigenicity were monitored daily by indirect ELISA as described in Appendix B.4.2.

2.2.4.7. Solvent stability

Except for chloroform and sodium dodecyl sulphate, which were used at a final concentration of 1% and 0.05% respectively, the effects of solvents on the particles were determined by mixing equal volumes of the solvent and purified particles followed by incubation at room temperature. These samples were examined daily for morphological changes by electron microscopy (Appendix B.5.1) and for antigenic changes by indirect ELISA (Appendix B.4.2). The following solutions were tested: acetone, methanol, $MgCl_2$ (50 mM), NaCl, (50 mM), KCl (50 mM), $CaCl_2$ (50 mM).

2.2.4.8. Thermal stability

Purified particles resuspended in PBS-$MgSO_4$ pH 7.2 were incubated at 40°C, 60°C and 80°C for 10 minutes. The effect of heat was monitored by examining changes in particle morphology and antigenicity by electron microscopy using negative staining and immunosorbent techniques (Appendix B5.1 and B.5.3).

2.3. Results and Discussion

2.3.1. Isolation and purification of the putative phage

2.3.1.1. Assessment of the efficiency of purification

The classical approach for assessing the efficiency of a bacteriophage purification protocol is to conduct infectivity assays and quantify the phage by doing plaque counts. With this particular putative phage, these tests could not be conducted due to the lack of a sensitive indicator strain, hence the use of electron microscopy to observe the structural integrity of the particles, and SDS-PAGE to monitor any contamination of the proteins associated with the particles through each step of the purification procedure.

2.3.1.2. Particle purification

The characterisation of the particles depended upon the ready availability of purified preparations. In developing the purification protocol, two major problems were encountered: low yield of particles, and the presence of a highly viscous, mucoid substance, most probably an extracellular polysaccharide (EPS). An indication of the low yield was the observation of very few particles by electron microscopy in purified preparations.

The major EPS produced by xanthomonads, xanthan, is readily separated from bacterial cells by high-speed centrifugation (Sutherland, 1993). This would explain the viscosity of the preparations obtained at this stage of the purification protocol. The presence of this viscous material prevented removal of contaminants by filtration through 0.22 μm, 0.45 μm or 0.8 μm Millipore filters. This substance also affected the final yield of the particles, following rate zonal centrifugation, by causing a proportion of the particles to aggregate with the EPS. This resulted in the sedimentation of the particles to the bottom of the centrifuge tube along with residual bacterial debris.

In attempts to decrease the amount of viscous material produced, the bacterial host was grown in liquid media (SMB, SMB with yeast extract, or nutrient broth). However, extremely large volumes of liquid cultures were required to produce the same quantity of particles as the cultures grown on solid media and there was only a slight difference in the viscosity of the resultant suspensions. This is not unusual since the poor multiplication of phages in liquid media has been well documented (Eisenstark, 1966).

In further attempts to decrease the viscosity, the bacteria were grown on SMB plates, and SMB containing yeast extract in concentrations varying from 0.01%-0.5%. The SMB and SMB with yeast extract-grown cultures produced suspensions that could be filtered - albeit with great difficulty - using 0.45 μm Millipore filters, indicating a decrease in the amount of viscous material. However, associated with this decrease in viscosity was a concomitant decrease in the yield of particles. It could be argued that this is to be expected when the bacterial host is subjected to the stress of nutrient limitation. However, it was noted that when grown on the eventual medium of choice (nutrient agar), the more mucoid the cultures, the greater the yield of the particles, and when subjected to CsCl density gradient centrifugation, the viscous material and the particles did not separate into individual bands. Although this could imply a possible association between the viscous material and the putative phage, it cannot be said with any certainty that the viscous material is a component of the particles. Only one phage, *Aerobacter aerogenes* phage K-2, is known to definitely possess a high molecular weight polysaccharide as an externally exposed constituent of the capsid (Oeltmann and Heath, 1975), while a few tailed phages are known to possess carbohydrates as minor constituents of their structure (Ackerman and DuBow, 1987). Alternatively, the viscosity may arise from a dependency of the putative phage on an EPS as

its receptor (Weiss *et al.* 1994). However, based on a comparison of the sedimentation coefficients of xanthan (14.8 x 10^6) and the particles (Section 2.3.3.), it appears that xanthan is not the putative EPS receptor in question. However, infection of *X. campestris* Gum^+ as well as Gum^- (xanthan negative) cells with the particles should be able to resolve the question of whether xanthan serves as a receptor for the binding of the putative phage.

Since attempts at decreasing the viscosity of the preparation at the propagation stage adversely affected the final yield, elimination of the viscous material by enzymatic digestion following particle isolation and clarification by differential centrifugation was investigated. Because the chemistry of the target substance was unknown, all enzymes with activity at a neutral pH were considered. The reason for this decision is that the extraction buffer had a neutral pH. Due to the lack of commercial availability of certain enzymes, such as endo-1,4-β-glucanases (xanthanases) and the low pH requirements of others, the effect of only α-amylase on the viscous material was investigated. The enzyme had a negligible effect on the viscosity, and its use was therefore discontinued.

The different buffers, viz. TEN, PBT and PBS pH 7.2, tested during the extraction procedure produced no visible differences in the final quality of the particle preparations. Therefore the use of the initial isolation buffer, PBS, was continued. The addition of 5 mM $MgSO_4$, - an additive which is required by some phages for maintenance of their structural integrity - to the buffer, produced samples which upon electron microscopic analysis, showed fewer particles penetrated by the stain (Figure 2.1A & B). This indicated that a greater number of particles had remained structurally intact in the presence of $MgSO_4$, hence the continued use of $MgSO_4$ as a buffer additive.

2.3.1.3. Isolation of intracellular particles

The absence of visible plaque formation led to the following questions: what proportion (if any) of mature putative phage particles remained within the cells at the time of harvesting? Could these intracellular particles serve as an additional source of material for further analysis? In an attempt to answer these questions, the bacterial cells were subjected to physical disruption by French pressing and sonication, and the particles were then purified as

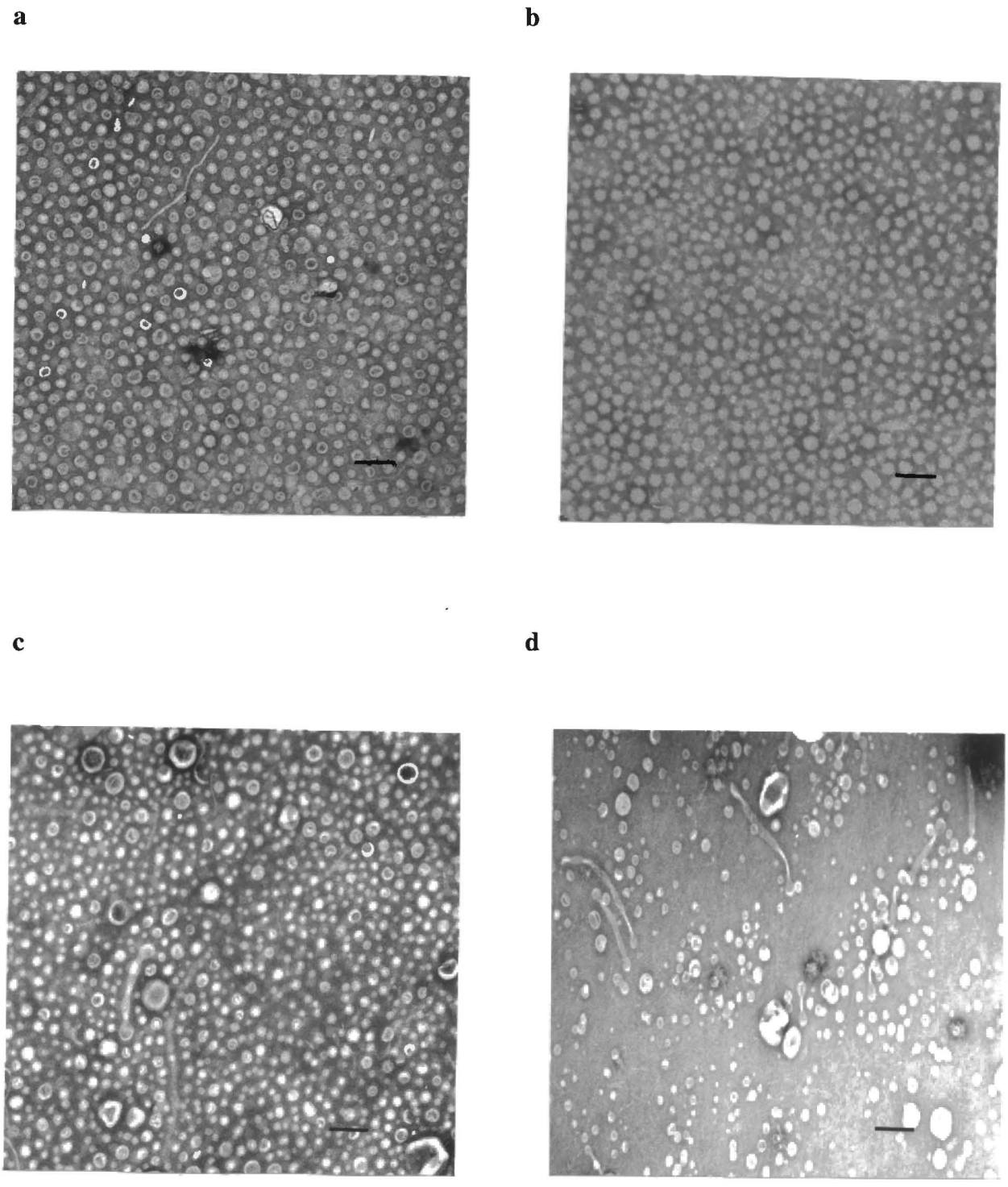


Figure 2.1. Assessment of the purification procedure by electron microscopy following the growth of the host on nutrient agar. (a) particles resuspended in PBS pH 7.2, (b) particles resuspended in PBS pH 7.2 containing $MgSO_4$, (c) particles concentrated by ultracentrifugation, and (d) particles concentrated by precipitation with polyethylene glycol, showing no defined shape. Magnification: 30 000x. The scale bar represents 150 nm.

described in section 2.2.2. The yield was compared to the extracellular fraction. These methods resulted in increased difficulty during the purification procedure as a consequence of the further increased viscosity of the solutions due to the intracellular debris. The samples obtained following French pressing and sonication were completely unlike those obtained without physical disruption. Although the samples contained a number of particles similar in shape and size to those obtained by purification of extracellular material, these particles were almost equalled in number by irregularly shaped particles. It was not apparent whether these latter particles were partially degraded particles or bacterial debris with sedimentation properties similar to that of the regular-sized particles. The fact that this debris could not be removed by further purification on sucrose gradients, rendered physical disruption ineffective at increasing the particle yield, and rendered the resultant preparations non-viable for use in accurate biochemical and biophysical analysis.

2.3.1.4. Assessment of methods for particle concentration

The efficiency of ultracentrifugation versus polyethylene glycol (PEG) precipitation in the concentration of the particles prior to rate zonal centrifugation was investigated. Ultracentrifugation proved to be most efficient with respect to the time required for concentration (2 hours, compared to 24 hours for PEG precipitation) and especially for the maintenance of the integrity of the particle structure (Figure 2.1c). The variability in the shape and size of the particles observed in the ultracentrifuged preparation may be due to the presence of entities other than the expected phage. For example, some of the particles may be outer membrane vesicles, which are certainly known to occur in a variety of sizes in Gram negative bacteria (Grenier and Mayrand, 1987). The phage appeared to be destabilised by precipitation with PEG. This was clearly evident from the profound morphological changes observed by electron microscopy of the particles. A number of particles appeared to possess long, flexuous tail-like structures, while many of the particles were infiltrated by the stain (Figure 2.1d).

Another method of concentration that was tried required 2 ml of 15% sucrose to be layered upon 4 ml of a 60% sucrose solution, with the rest of the tube was filled with the supernatant obtained after the initial low speed centrifugation step, followed by centrifugation at 23 000 rpm for 8 hours. The principle of this method is that all contaminants will remain above the 15% sucrose, while the putative phage will partition at the 15-60% sucrose interface. This

method was deemed inefficient with respect to the yield of particles, which was only slightly higher than that obtained from 5-20% gradients, and the time required to obtain a purified sample suitable for further analysis: this was 8 hours, plus 2.5 hours for a second sucrose gradient to eliminate most of the bacterial contaminants picked up upon removal of the phage band from the 15-60% gradient by tube puncture.

2.3.1.5. Sucrose gradient centrifugation

Initially, the preparations were purified on a 10-40% sucrose gradient centrifuged at a speed of 23 000 rpm for 2.5 hours in a Beckman SW27 swinging bucket rotor. However, this was not ideal for separation of the particles, since the sample layered onto the gradient sedimented no more than 1 cm down the length of the gradient and still contained a small amount of bacterial debris. After 4 hours, the sample had sedimented through $^{1}/3$ of the gradient. This same result was achieved using a 5-20% gradient centrifuged at 25 000 rpm for 2.5 hours, hence the continued use of the latter type of gradient.

2.3.2. Particle morphology and physical characteristics

Electron microscopy of preparations negatively stained with 2% (w/v) uranyl acetate revealed particles hexagonal in outline and icosahedral in shape (Figure 2.2). The variability in particle size is illustrated in a frequency histogram (Figure 2.3) from which the average particle diameter was calculated to be 33.5±4.5 nm. The observed variability in size might possibly be due to less than optimal isolation and purification procedures rather than a mixture of different particles. If so, then further optimisation of the phage isolation and purification methods would certainly be required. Notably absent was any sort of a tail structure extending from one of the vertices.

The possibility that a tail may have been lost during the process of purification, or the preparation of samples for electron microscopy using 2% uranyl acetate, was considered. However, examination of samples taken at different stages of the purification procedure (including the very first stage of washing the nutrient agar plates with buffer) using the various buffers and methods mentioned in Section 2.3.1, did not reveal any change in the morphology of the particles. Neither did the use of an alternate stain, methylaminetungstate. Thus the absence of a tail appears to be a genuine feature of the particles and not an artefact

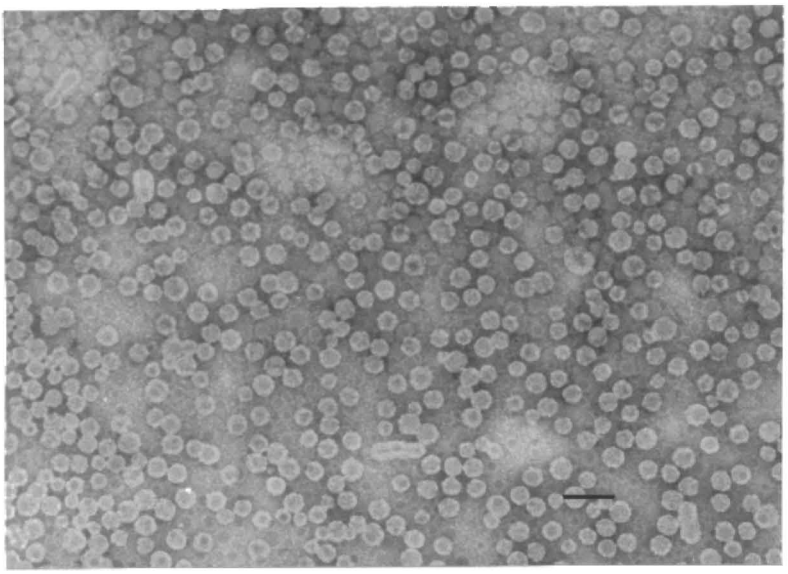


Figure 2.2. Electron micrograph of a purified preparation showing the hexagonal morphology of the particles. Magnification: 30 000x. The scale bar represents 100 nm.

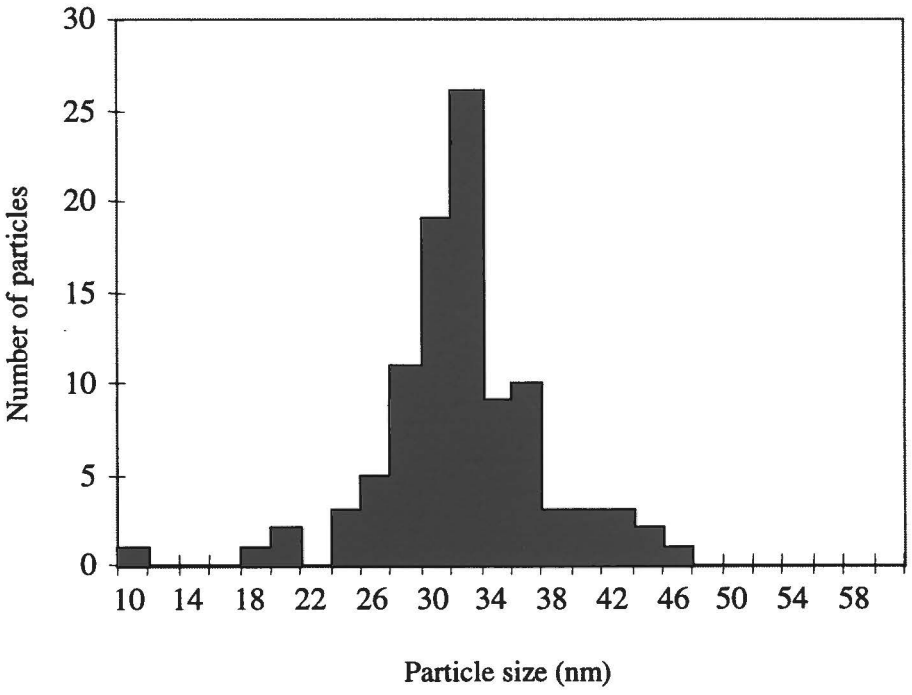


Figure 2.3. Frequency histogram illustrating the range in particle diameter.

produced by the process of sample preparation. This is therefore, a distinguishing feature of the particle, as all icosahedral phages described for Xanthomonas can be classified as morphotypes A1, B1, B2 and C1, corresponding to the families of tailed phages *Myoviridae, Siphoviridae* and *Podoviridae* (Ackerman, 1996).

When subjected to caesium chloride density gradient centrifugation, the sucrose gradient purified particles produced a single light-scattering band. The particles in this band, identified by EM and SDS-PAGE, had a buoyant density of 1.347 g/ml (4 determinations) compared to 1.35 g/ml for BMV.

The sedimentation coefficient is a function of a particles' size as well as its density, and is an indication of the velocity at which a molecule sediments in a centrifuge. The relative sedimentation coefficient of the particles at a neutral pH was 85S, corresponding to the $S^{o}_{20,w}$ of the internal standard, BMV. Both particle types possessed the same approximate sedimentation coefficient despite the difference in particle size - 25 nm for BMV as opposed to the 33.5±4.5 nm of the putative phage - implying that the inherent properties of the latter other than size, *i.e.* its chemical composition, must influence the slow sedimentation rate of these particles. Considering the empirical equation relating the sedimentation coefficient and molecular weights of phages, derived by Pitout *et al.* (1969):

$$S^{o}_{20,w} = 1.114 \times 10^{-3} \times M^{0.729},$$

the hypothetical molecular weight of the phage was calculated to be 4.98×10^{6}.

2.3.3. The nature of the particles

A distinctive characteristic of any particular group of viruses is the number and molecular weight of the structural proteins comprising the capsid and tail (if present). SDS-PAGE of purified particle preparations consistently produced three major bands and two minor bands (Figure 2.4b). The major proteins have molecular weights of 94, 32 and 21.8 kDa, while the molecular weights of the two minor proteins corresponded to 40 kDa and 25.7 kDa. The densitometer scan of the stained gel produced peaks corresponding to the three major and two minor proteins (Figure 2.4a) and allowed for the calculation of the percentage areas beneath the peaks (Table 2.1.). These results provided further insight into the protein composition of the particles by indicating the relative proportions of each of the five proteins

associated with them. The densitometer results showed that the 94, 32 and 21.8 kDa proteins are the major constituents of the particles (Table 2.1.).

IEB tests demonstrated that the 40 kDa protein, which constitutes only 9.8% of the total protein content of the phage, is the most immunogenic component, followed by the 32, 94; 25.7 and 21.8 kDa proteins respectively (Figure 2.5a). Based on these results one may propose a more exposed position on the surface of the particle for the 40 kDa protein.

The distinctive shape, *i.e.* the icosahedral shape and absence of a tail, and the slight difference in size of the particles observed by EM, as well as the absence of plaques on solid media, prompted us to establish that the particles were not the products of vesiculation of the bacterial membrane. Since the vesicles originate from bacteria, they would naturally have protein compositions similar to that of the bacteria. Zhou *et al.* (1998) and Kadurugamuwa and Beveridge (1995) showed that vesicles produced by *Porphyromonas gingivalis* and *Pseudomonas aeruginosa*, respectively, produced banding patterns essentially similar to that of outer membrane (OMP) and whole-cell extracts, although fewer proteins were present in the vesicles.

Since an antiserum to the purified particles had been produced, IEB was used to determine whether there was any similarity between the bacterial and putative phage proteins. It was demonstrated that the antibody raised against the sucrose gradient-purified product reacted with both the bacterial sample and purified particles. However, the banding patterns were completely different (Figure 2.5). The reaction of the antiserum with components from both the particles and bacterium is almost certainly due to contamination of the sample injected into the rabbit by traces of PCB 22 components, resulting in a positive reaction with the antibody produced. PCB 22, itself, was not included in this experiment due to the production of high background that obscures the presence of distinct, individual bands. Therefore, on the basis of the IEB analysis, it was determined that the particles observed by electron microscopy were probably not outer membrane vesicles of bacterial origin.

2.3.4. Isolation and characterisation of the particles' nucleic acid

To determine the nature and size of the putative phage genome, the nucleic acid was isolated from sucrose gradient-purified samples. The method used most consistently is described in

Section 2.2.45a. However, a number of other methods were attempted, since the yield obtained from the aforementioned method was not very high or very consistent. Standard procedures developed for the isolation of DNA from bacteriophages λ and M13 (Sambrook *et al.*, 1989) were unsuccessful. Formamide extraction (Ausubel *et al.*, 1989), treatment with lysing solutions containing 2-10 mM EDTA (in 20-50 mM Tris-Cl pH 8 and 150 mM NaCl) and 2-3% SDS produced very low yields ranging from 20 ng - 1 µg per purified particle preparation. In addition, these methods were frequently unsuccessful. Hence they were not used as the preferred method of extraction.

The DNA of several bacteriophages is known to be covalently linked to proteins (Weiss et al. 1994; Garcia et al. 1985; Grimes and Anderson, 1989). Since such an association could result in a significant loss of nucleic acid from the aqueous phase of the extraction solution, 100-400 µg/ml of proteinase K was added to the particles in lysing solution. However, no appreciable difference was observed in the final yield obtained. More successful was the method of Kado and Liu (1981) for the isolation of high molecular weight plasmids.

The method requires the use of a high pH lysing solution. This method, successful on a few occasions, produced yields of 0.3 µg-2 µg per 240 µg of purified phage preparation. However, shortly after attempting this method, despite there having been no change in the conditions of particle isolation and purification, successful isolation of the nucleic acid using any of the methods mentioned, became increasingly rare.

The nucleic acid of the particles was identified as single-stranded DNA on the basis of the following criteria: (i) the nucleic acid was sensitive to DNaseI, but resistant to RNase A and all the restriction endonucleases tested; (ii) it was sensitive to the single-strand specific SI nuclease; (iii) it stained bright red after treatment with Na2HPO4 and pale green in the presence of tartaric acid in accordance with Bradleys' (1966) method for fluorescent staining of phage nucleic acids. Further evidence of the single-stranded nature of the putative phage genome was provided by the fact that the DNA could be labelled by random priming for hybridisation purposes without prior denaturation.

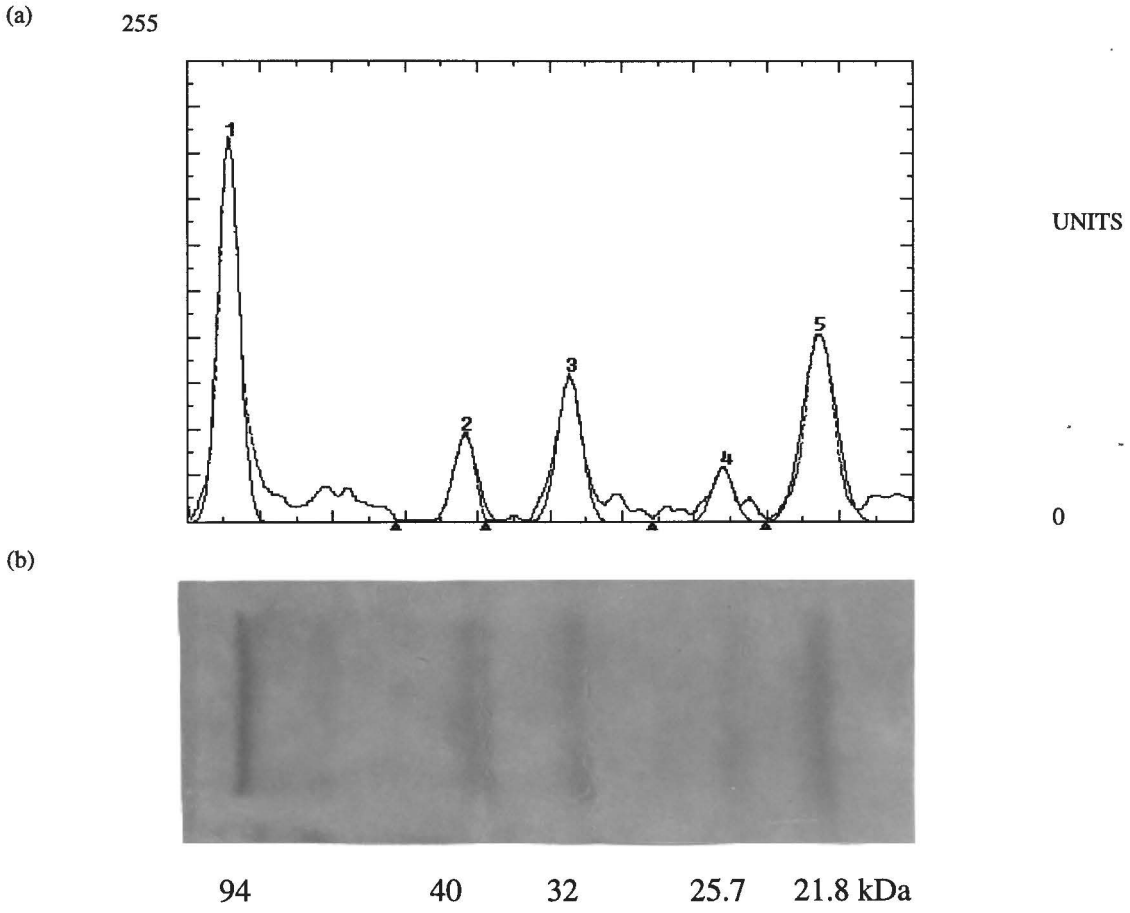


Figure 2.4. SDS-PAGE and densitometer scan of the purified particle proteins. (a) densitometer scan of the particle proteins, with peaks numbered 1-5 corresponding to the protein bands (b) the five particle proteins fractionated by SDS-PAGE, with their molecular weights indicated below.

Table 2.1. Percentage area differences of the five particle proteins

Protein size	Percentage area
94 kDa	38.4
40 kDa	9.8
32 kDa	16.9
25.7 kDa	5.7
21.8 kDa	29.2

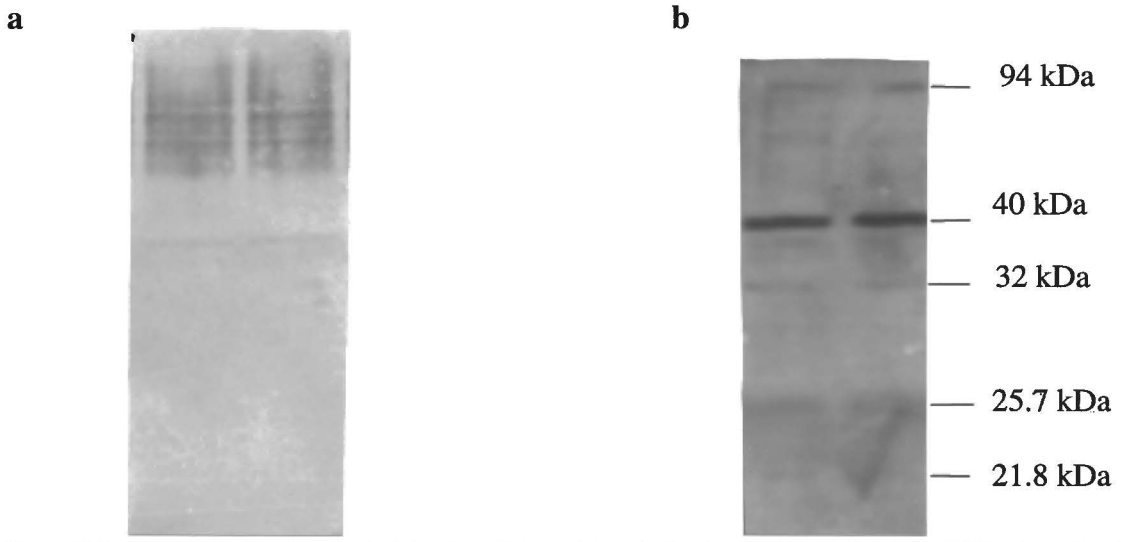


Figure 2.5. The reactivity of the bacterial and particle proteins electrophoresed through a 12% SDS-polyacrylamide gel to the antiserum raised against a purified particle preparation. a: *X. campestris* pv. *vasculorum*, b: purified particles

The size of the DNA isolated from the purified particles could not be accurately determined due to its single-stranded nature, which made it resistant to endonucleases. An estimation of the size could probably have been obtained from a comparative study with λ DNA electrophoresed on a denaturing gel, however, due to the difficulty in isolating sufficient DNA from the particles, this experiment was not performed. Given the single-stranded nature of the DNA, it was assumed that replication would occur via a double-stranded replicative intermediate. Therefore, the host PCB 22 was subjected to standard protocols for plasmid isolation. This resulted in the isolation of a high molecular weight extra-chromosomal element, which upon Southern hybridisation, was shown to hybridise to the 32P-labelled single-stranded particle-derived DNA (Figure 2.6). This result led to the hypothesis that the extra-chromosomal DNA was probably not a plasmid.

The putative replicative form (RF) DNA had recognition sites for the following restriction endonucleases: BamHI, BclI, BglI, Bst XI, ClaI, EcoRI, EcoRV, HaeIII, HindIII, NcoI, NotI, PstI, PvuI, PvuII, SacI, SalI, StuI, StyI and XhoI; however, it was resistant to digestion by the endonucleases KpnI, MluI and XbaI. The average size of the putative RF DNA was estimated to be 53.9 kb following digestion by EcoRI and SalI (Figure 2.7). In most viruses, capsid size places rigorous constraints on the amount of DNA that can be packaged: for example, bacteriophage λ which has a head diameter of 60 nm and a genome size of 48.5

kbp, is able to package 46-54 kbp of DNA; similarly T4 phage heads, 111 nm long and 78 nm wide, are able to package 336 kbp of DNA (Murphy et al., 1995). Since the diameter of the putative phage is 33.5±4.5 nm - approximately half the size of λ - one would expect the genome to be considerably smaller than that of λ. However, in contrast to the double-stranded nature of the λ genome, the DNA isolated from the purified particles was demonstrated to be single-stranded, a fact that could considerably increase the amount of DNA which can be packaged. In addition, ssDNA molecules have a strong tendency to fold back and form hydrogen bonds between complementary nucleotides, which would result in the formation of double-helical hairpin loops and ultimately more compact molecules (Watson et al., 1987). Nevertheless, even taking all of the aforementioned statements into account, the putative RF DNA appears too large to be packaged as ssDNA in a single particle the size of those isolated. Moreover, the particles would be considerably denser than those of brome mosaic virus – which contain approximately 30% (w/w) ssRNA for a particle mass of 5.4 x 106 (Rybicki, 1995) - instead of being the same density, if all of the DNA were packaged.

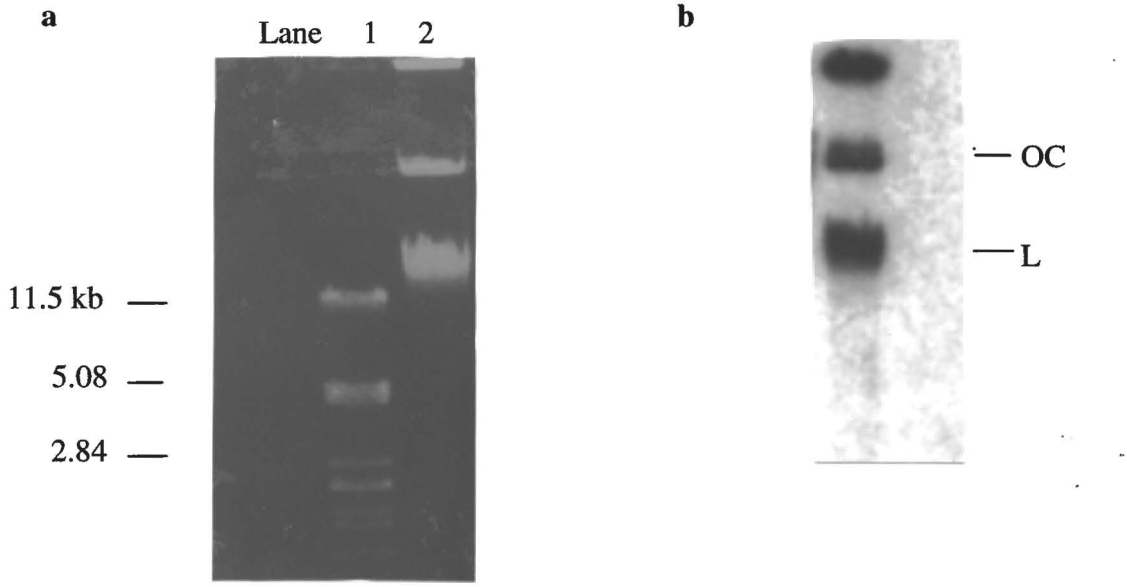


Figure 2.6. Southern blot analysis of undigested putative phage replicative form DNA isolated from PCB 22 (a) ethidium bromide-stained gel. Lane 1: λ-*Pst*I, Lane 2: putative RF DNA (b) gel from (a) transferred to nylon membrane and hybridised with ^{32}P-labelled virion DNA. OC: open circular, L: linear

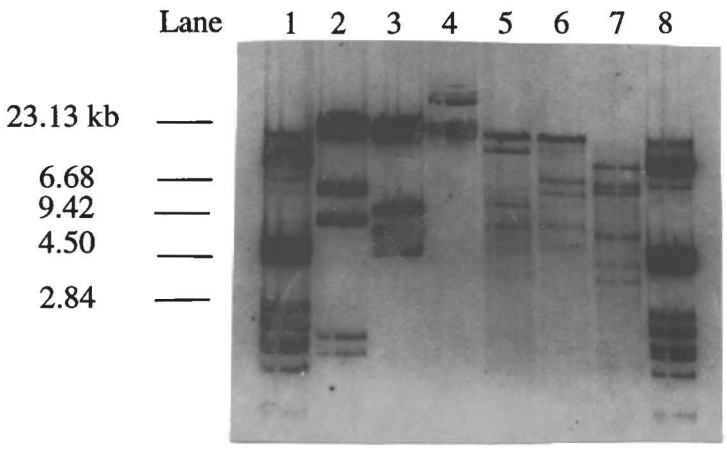


Figure 2.7. Analysis of putative RF DNA digested with restriction enzymes, *Hin*dIII (Lane 5), *Sal*I (Lane 6) and *Eco*RI (Lane 7). Molecular weight makers: λ-*Pst*I (Lane 1 & 8), λ-*Eco*RI (Lane 2) and λ-*Hin*dIII (Lane 3). Lane 4: undigested RF.

2.3.5. Particle stability

The effect of temperature, solvents and buffers of different pH on the infectivity of a virus give a clear indication of the stability of that virus, i.e. the integrity of the capsid and genome. However, since it was not possible to determine particle stability in this manner due to the unavailability of a particle-free host and hence the lack of an infectivity assay, the effect of pH and solvents were monitored by changes in the antigenicity and morphology of the particle. The particles were stable in PBS-MgSO4 pH 7.2 and could be stored at 4°C for approximately 12 months without appreciable changes in antigenicity and morphology. After 2 hours at pH 5, 6 and 8 there was only a 2-5% decrease in the antigenicity as determined by ELISA relative to particles resuspended in PBS-MgSO4 pH 7.2, whereas pH 4, 9 and 10 showed an 11-13% decrease (Figure 2.8a). With respect to antigenicity, the particles remained very stable at pH 8, becoming less reactive only after 3 days. The particles incubated at pH 4, 5, 6, 9 and 10 became progressively less reactive with time. This was particularly evident at pH 4, followed by pH 10.

The changes in morphology were observed by electron microscopy after 4 days. A decrease in the number of particles at pH 5 and pH 6 relative to pH 7.2, was clearly evident, while an increase in particle size was observed at pH 4, pH 9 and pH 10 (Figure 2.8b). The observed particles were 1.7 - 2.4x larger than particles incubated at pH 7.2, with every single particle penetrated by stain. This is a clear indication of the adverse effect these pH's have on the structural integrity. In fact, the variability in the size of particles at pH 7.2 may be indicative of a sub-optimal buffer pH.

The particles exhibited a dramatic decrease in antigenicity after 4 days of incubation in 0.05% SDS and 50 mM CuSO4 (Figure 2.9). The particles in these samples had approximately 40% the immunogenic reactivity of those incubated in PBS-MgSO4 pH 7.2 and very few particles (intact or disrupted) were observed by electron microscopy after 4 days. The sharp decrease caused by exposure to SDS may be explained by the interaction of SDS with the proteins, resulting in the destabilisation of the putative capsid. Particles remained relatively stable in the presence of the monovalent salts NaCl (result not shown) and KCl, as well as the divalent $CaCl_2$ (Figure 2.9). The presence of methanol appears to be tolerable for at least 2 days indicating that the particles are resistant to a certain extent.

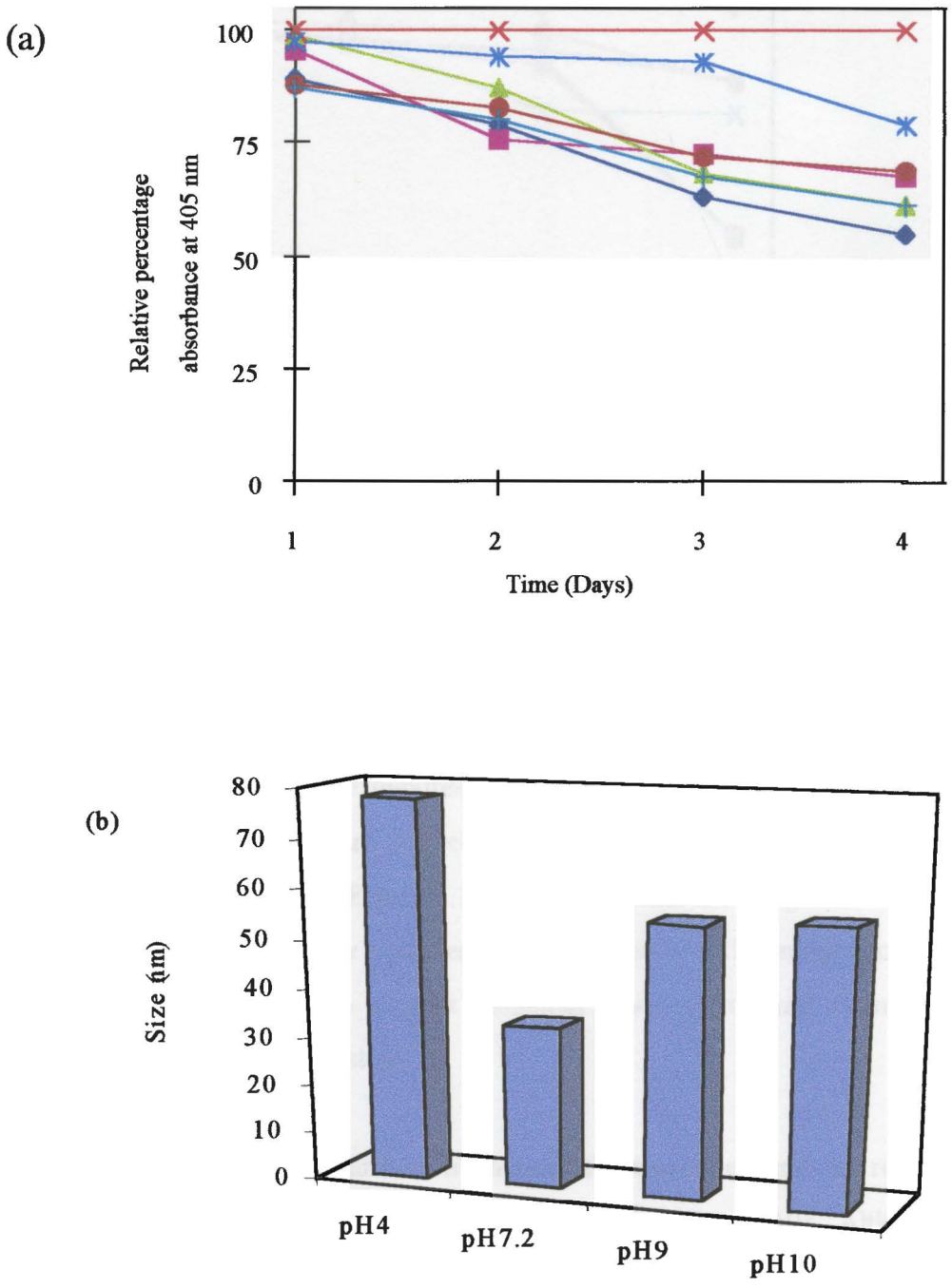


Figure 2.8. The effect of pH on the particles with respect to: (a) antigenicity as determined by indirect ELISA. The relative absorbance was calculated as the percentage of the absorbance of 0.5 μg/ml of particles resuspended at pHs 4, 5, 6, 8, 9, and 10 relative to the absorbance of 0.5 ?g/ml of particles resuspended in PBS-$MgSO_4$ pH 7.2. (b) morphology as determined from measurements of particle size on the negative film of electron micrographs.

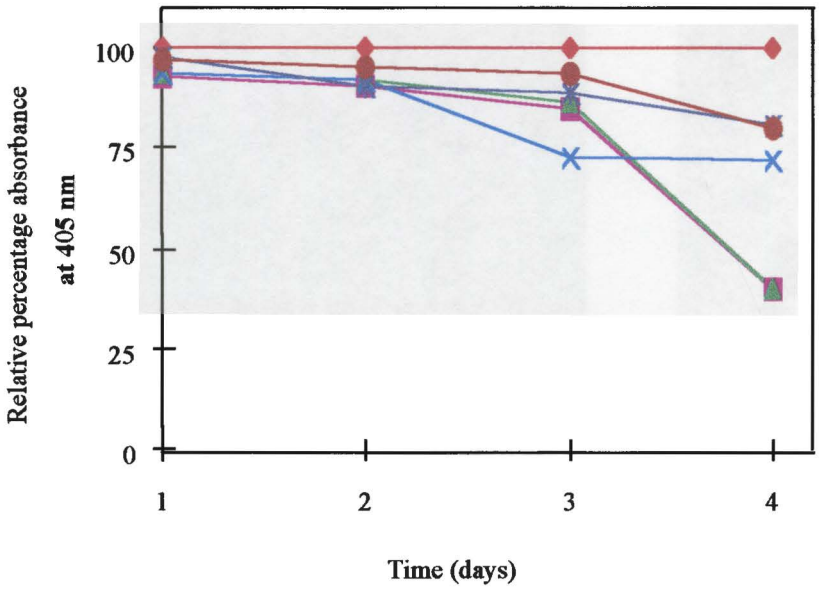


Figure 2.9. The effect of solvents on the particles as a function of antigenicity, monitored by indirect ELISA. The relative absorbance was calculated as the percentage of the absorbance of 0.5 μg/ml of particles resuspended in PBS-$MgSO_4$ pH 7.2.

Electron microscopy after 30 minutes of incubation at 30°C in 1% chloroform showed a decrease in the number of particles relative to the control and an increase in the number of particles penetrated by stain (Figure 2.10), indicating that the phage is sensitive to chloroform to a certain degree. Very few particles were observed after 30 minutes of acetone treatment (result not shown). Those present exhibited severe structural changes in addition to penetration of the particle by stain.

Upon exposure to heat, the particles maintained their structure and still reacted very strongly with the antiserum at temperatures of 40°C and 60°C (Figure 2.10). Although there was still a relatively strong reaction at 80°C, many of the observed particles were structurally defective, i.e. a variety of shapes were observed (result not shown). Some of the antibody-binding might also be the result of recognition of proteins in their denatured form. Hence, the fuzzy, unclear images when the 80°C-treated sample was viewed by EM.

In summary, what we have isolated is a 33.5±4.5 nm particle with a seemingly unique morphology for a Xanthomonas phage. The particles, despite being significantly larger than BMV virions, with the same buoyant density, had the same sedimentation rate. This is abnormally low, and could possibly be attributed to the presence of the viscous material.

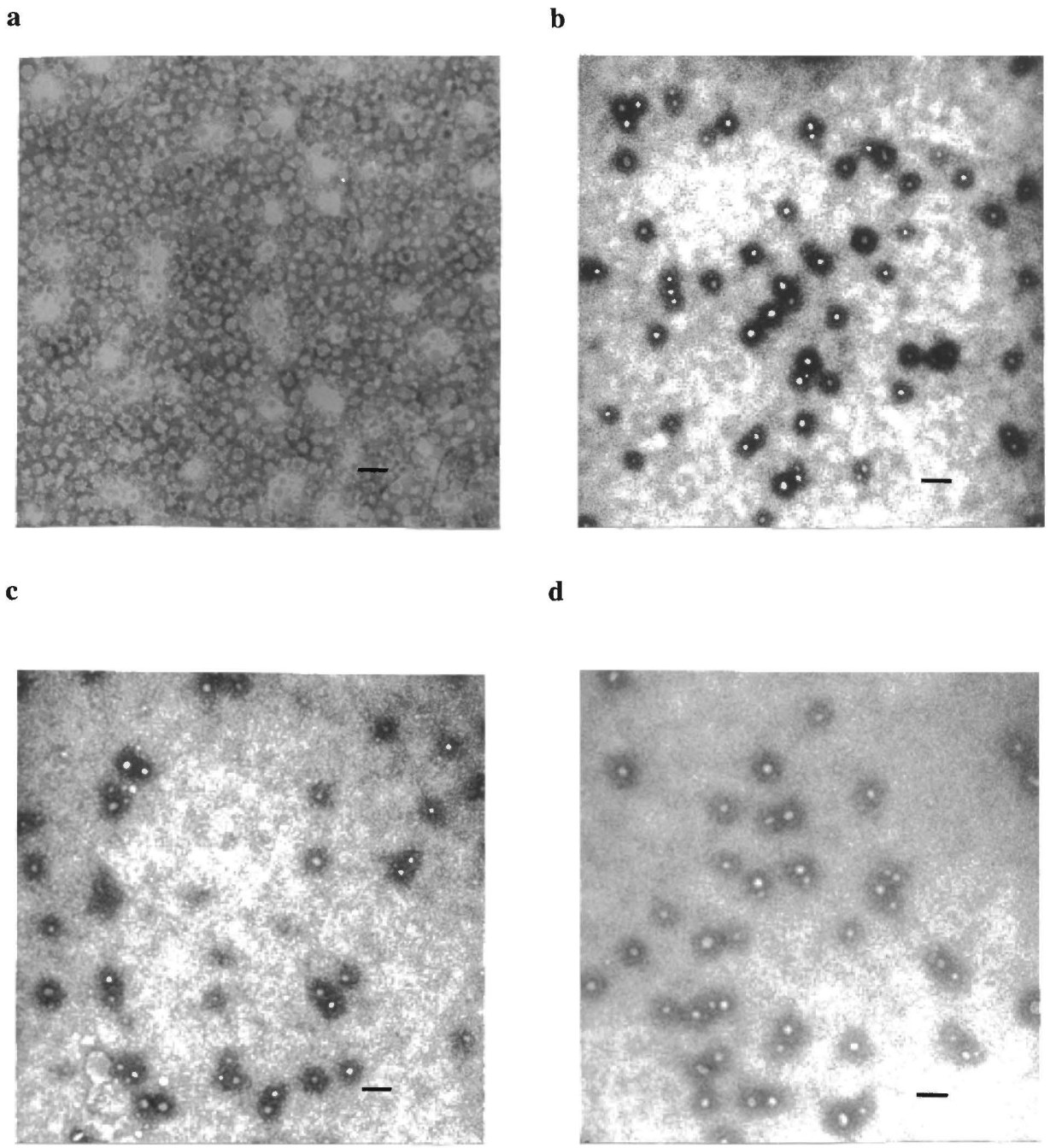


Figure 2.10. Electron micrographs of the purified particles. Magnification 30 000x. The scale bar represents 100 nm.

(a) the particles incubated in 1% chloroform for 30 min and negatively stained in uranyl acetate

(b) ISEM of particles resuspended in PBS-$MgSO_4$

(c) ISEM of particles exposed to a temperature of 40°C for 10 mins

(d) ISEM of particles exposed to a temperature of 60°C for 10 mins

Based on the results obtained from IEB analysis, the possibility of these particles being vesicles produced by budding of the bacterial outer membrane, was ruled out. It was therefore concluded that the particles were most probably of bacteriophage origin, and since further evidence to support this conclusion is presented in chapter 3, the particles will henceforth be referred to as bacteriophages. Their consistent association with ssDNA, even if this is smaller than what appears to be virus-related dsDNA isolated from bacterial cells, strengthens this conclusion.

On the basis of its gross morphology, *i.e.* the hexagonal shape and lack of tail, this phage can only be classified as morphotype D2. The absence of a tail raises the interesting question of how this phage infects its bacterial host.

The small icosahedral phage ϕX174 has apical spikes which are involved in adsorption, whereas small, tailless RNA phages such as MS2, inject their nucleic acid via the F pilus. Electron microscopy of the host, PCB 22 (Figure 1.1), showed the phage to be associated with the cell wall, but whether these particles are actually attached to specific receptors located on the bacterial surface - by some unknown mechanism, as it lacks the usual appendage for attachment - is uncertain.

Evidence of a modified vertex that could be involved in adsorption was not apparent from any of the numerous samples prepared for electron microscopy. Thus the question of how the phage infects bacteria, remains to be answered

The phage is a very stable particle with respect to pH and solvents, but only relatively stable at elevated temperatures. The novel phage appears to be in a class of its own with respect to its combination of morphology and nucleic acid content; however limited data concerning the physicochemical characteristics of xanthomonad phages are available. Hence, few comparisons can be made between this phage and other phages known to infect xanthomonads. However, its pH stability (determined by EM and ELISA) is comparable to that of Xp12 (determined from infectivity tests), a tailed phage of *X. oryzae* (Kuo *et al.* 1968a). Its thermal stability within the range tested is much lower than the 85°C required by phage Xf for inactivation (Kuo *et al.* 1969) and is closest to phage Xp12 which has a thermal inactivation temperature of 66°C (Kuo *et al.* 1968a).

Not only does this phage have a novel morphology that does not fit that of any of the accepted phage families, but the fact that it also has a single-stranded genome, further prevents its classification at this particular time. But then, new types of viruses with "unusual" properties are continually being isolated as analytical techniques improve and research interests broaden. Thus, the current classification system would have to be adapted in order to accommodate these viruses with new properties.

CHAPTER 3

The Biological Properties of the Bacteriophage

Contents

The Biological Properties of the Novel Bacteriophage

Summary

An investigation into the biological properties of the novel bacteriophage showed that it is a temperate phage, which does not produce visible plaques on solid media and is not inducible by UV or mitomycin C. Electron microscopy and anti-phage serum showed that the phage is present in all isolates of the chocolate spot pathogen and that similar particles are present in *Xanthomonas campestris* pathovars *campestris*, *abberans*, *armoraciae* and *raphani*. The serological relationships between the novel phage and and two *Xanthomonas* tailed phages RR68 and HT_{3h} were investigated using indirect ELISA, immunoelectroblotting and immunosorbent electron microscopy. No serological relationship was detected between the novel phage and RR68. The phage antibodies recognised three proteins of molecular weights 21.8, 13.79 and 12.64 kDa in HT_{3h}. Phage-like particles were detected in the host PCB 22 by electron microscopy following ultrathin-sectioning of the bacteria. Nucleic acid hybridisation could not confirm the integration of the double-stranded form of the phage genome into either the host chromosome or the extrachromosomal element.

3.1. Introduction

Phages can be detected by a number of methods, the most obvious being the observation of plaque formation in bacterial lawns on solid media. The morphology of plaques can often give an indication as to the identity of a phage or class of phages. However, this method is only applicable to phages capable of lysing their hosts. Temperate phages engaged in the lysogenic life cycle can thus remain undetected until such time when a bacterial strain sensitive to the phage is discovered, or the lytic cycle is induced by prevailing environmental conditions.

Despite the fact that lysogeny is regarded as a very stable state, free phage particles exist in lysogenic cultures as a consequence of a small, random fraction of the lysogenic population which have expressed their ability to release phage (Hayes, 1964). However, the number of infectious particles produced is often so low that lysogeny may only accidentally be recognised when viewing the bacteria with the aid of an electron microscope. Because of the stability of the lysogenic state, the probability of lysis and phage release under standard conditions is very low. This probability is increased when lysogenic bacteria are induced to release phage by external factors such as ultraviolet (UV) radiation and chemical mutagens, *e.g.* mitomycin C and fluoropyrimidines. These factors either damage DNA (UV) or inhibit DNA synthesis (mitomycin C). As a result, the RecA protein, part of the bacterial DNA repair system, is activated, cleaves the phage repressor protein and thus induces the prophage to enter the lytic cycle (Prescott *et al.*, 1990). However, not all temperate phages are inducible (Barksdale and Arden, 1979).

Having identified the existence of an apparently temperate phage by electron microscopy, it was essential to obtain evidence more conclusive than the lack of plaque formation, in order to conclusively classify it as a temperate phage capable of lysogeny. This necessity stems from the discovery of pseudolysogeny, a state regarded as a variation of true lysogeny. Pseudolysogeny is described as a persistent viral infection or a carrier state. In this state, bacterial populations are composed of infected (resistant) as well as uninfected (sensitive) cells, with the progeny of either one of these types of bacteria having the probability of being sensitive or resistant (Barksdale and Arden, 1974; Hayes, 1964). Thus pseudolysogeny is not a stable state in which the ability to produce phage is faithfully inherited by the daughter cells. Unlike true lysogeny, the persistence of pseudolysogeny within a population is

dependent upon the presence of free exogenous phage, which subsequently infect and lyse sensitive bacteria. As a result, it is possible to distinguish between pseudolysogeny and true lysogeny by growing the bacteria in the presence of phage antiserum that will inactivate free particles, thereby eliminating phage infection within a population.

Although particle morphology is often a distinguishing feature, additional evidence is required in order to meet the strict criteria of the taxonomic classification system, thereby determining the relationship of the new phage with other phages. This evidence can take the form of a study of the biophysical and biochemical characters of the phage (as described in Chapter 2) as well as an investigation into its biological properties. The latter category includes the study of phage host range, serological relationship with other phages; mode of existence, *i.e.* as an episome or a chromosomally integrated prophage; as well as cytology, *i.e.* the location of the phage particles within the host prior to release. In this chapter, electron microscopy, serological techniques and nucleic acid analysis were employed to elucidate the possibly temperate nature of the phage and investigate the above-mentioned biological properties.

Infection of *X. c.* pv. *oryzae* with the filamentous phage Xf is known to cause considerable changes in the properties of the host (Kamiunten and Wakimoto, 1982). Not only does Xf infection cause severe changes in intracellular structure, vesiculation as well as retardation of bacterial growth at the optimal growth temperature of 30°C, but it also leads to bacterial tolerance to an elevated temperature of 35°C. Furthermore, infected bacteria produced greater quantities of EPS and incited more severe disease symptoms on rice, than uninfected *X. c.* pv. *oryzae*. In order to conduct such studies on the novel phage, an uninfected, sensitive isolate of the chocolate spot pathogen was required. Hence, another objective of this project was to identify an isolate of the chocolate spot pathogen that did not harbour the phage and was sensitive to infection by the phage, thus facilitating comparative studies that would elucidate the nature of the phage-host relationship.

3.2. Materials and Methods

3.2.1. Bacterial and bacteriophage sources

The bacteriophages other than the novel phage as well as the bacterial isolates used in this study are listed in Table 3.1. Working cultures of the bacteria were maintained by regular subculturing on nutrient agar Biolab). Long term storage of bacteria was carried out in 20% glycerol at -20°C.

3.2.2. Bacteriophage propagation and purification

The novel phage was purified as described in Section 2.2.2. The lytic phages RR 68 and HT_{3h} were propagated by infecting their respective phage-free hosts, *X. c.* pv. *campestris* ATCC 33437 and *X. c.* pv. *campestris* ATCC 33442. The bacteria were assayed for plaque formation on phosphate buffered tryptone (PBT) agar (Appendix A.4) using the double layer agar method, following an 18-24 hour incubation at 30°C. The phages were harvested by washing the plates with 5 ml phosphate buffered tryptone (PBT) for 2 hours with occasional shaking (Liew and Alvarez, 1981a). The suspensions were subjected to differential centrifugation to remove bacterial debris. Following the final low speed centrifugation step, the pellet was resuspended in PBT and stored over 1% chloroform at 4°C.

3.2.3. Electron microscopic (EM) analysis by negative staining

In order to find a phage-free isolate, the bacteria listed in Table 3.1 were first examined by electron microscopy. The bacteria were grown for 2 days at 30°C on nutrient agar and the plates were washed with 5 ml PBS-$MgSO_4$ pH 7.2 for 2 hours. The suspensions were subjected to low speed centrifugation to remove most of the bacterial cells. The supernatants were then prepared for EM using the negative staining method (Appendix B.5.1).

Table 3.1. Bacteria and bacteriophages used in this study

Isolates	Origin
X. campestris (*X. c.*) PCA 11	KwaZulu-Natal[a]
X. c. PCA 12	KwaZulu -Natal
X. c. PCB 22[b]	KwaZulu -Natal
X. c. PCB 31	KwaZulu -Natal
X. c. PCB 32	KwaZulu -Natal
X. c. PMZ 131	KwaZulu -Natal
X. c. PMZ 232	KwaZulu -Natal
X. c. PMZ 331	KwaZulu -Natal
X. c. pv.*vasculorum* 5757	NCPPB[c]
X c. pv. *campestris* 528[T]	ATCC[d]
X. c. pv. *aberrans*	ATCC
X. c. pv. *armoraciae*	ATCC
X. c. pv. *graminis*	ATCC
X. c. pv. *raphani*	ATCC
X. c. pv. *campestris* 33442	ATCC
X. c. pv. *campestris* 33437	ATCC
Bacteriophage RR 68	ATCC
Bacteriophage HT_{3h}	ATCC

[a] isolates provided by Mark Laing, Univeristy of Natal, Pietermaritzburg, South Africa (Kariem, 1994).

[b] designated type strain of the chocolate spot pathogen.

[c] National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom.

[d] American Type Culture Collection.

[T] Type strain for the species *Xanthomonas campestris*

3.2.4. Immunological assays

3.2.4.1. The effect of antiserum on phage production

One hundred microlitres of a log phase culture of PCB 22 was incubated with an equal volume of anti-phage serum at room temperature for 15 mins. The cells were precipitated by centrifugation and used to inoculate nutrient broth containing 5% phage antiserum, which was then incubated at 30°C for ~18 hours. The culture was subsequently diluted 1:100 into antiserum-containing nutrient broth and incubated at 30°C for a further 18 hours. This process was repeated once more. The final culture obtained was prepared for EM (Appendix B.5) in order to determine whether the phage production persisted.

3.2.4.2. Immunoelectroblot (IEB)

The IEB tests were used as an alternative method of detection for the phage in bacterial cultures. They were also used to determine the serological relationships between the novel phage, the particles observed by EM of the chocolate spot isolates and the phage-like particles observed for *X. campestris* pathovars *campestris* ATCC 528, *aberrans*, *armoraciae* and *raphani*. The samples were prepared by subjecting the cultures to differential centrifugation in order to minimise the background caused by bacterial proteins. The pellet obtained after differential centrifugation was resuspended in PBS-$MgSO_4$ pH 7.2. For all the samples investigated, 20 µg of protein was disrupted (Appendix B.3.2) and electrophoresed through a 12% polyacrylamide gel as described earlier. The proteins were electroblotted to nitrocellulose membranes (pore size 0.45 µm), the membranes were probed with the antiserum raised against the phage (Section 2.2.2.) and detected by colour reactions (Appendix B.4.1).

3.2.4.3. Indirect ELISA

Indirect ELISA, as described in Appendix B.4.2, was used to determine the serological relationship between the novel phage and the two *Xanthomonas campestris* pv. *campestris* phages, RR 68 and HT_{3h}. Calibration titrations with dilution series of antigen, primary and secondary antibody were first performed in order to obtain sensitive detection with minimum background.

3.2.4.4. Immunosorbent electron microscopy (ISEM)

ISEM was used to (i) enrich for particles, (ii) determine the reaction of the antibody with the structure of HT_{3h} (Appendix B.5.3). The novel phage antiserum was used in this study. The grids were viewed using a ZEISS EM 109 electron microscope and photographed at 80 000 x magnification.

3.2.5. Assays for immunity and phage infectivity

Single colonies of all the bacteria listed in Table 3.1 were transferred to nutrient broth and incubated for 18-24 hours at 30°C. To assay the immunity of these cultures, 0.1 ml of the bacterial samples were mixed with soft agar and poured onto a nutrient agar plate. The novel phage (10 µl) was spotted onto the surface of the layer containing the bacteria to be tested. Liquid cultures of *X. c.* pv. *vasculorum*, *X. c.* pv. *graminis*, *X. c.* pv. *campestris* ATCC 33437 and 33442 were also grown in the presence of the novel phage and subsequently monitored for the presence of phage by EM. Phages RR 68 and HT_{3h} were only spotted onto the chocolate spot isolates and their respective hosts. Sensitive cells formed a clear zone of lysis, while immune cells did not. For quantitative determination of phage RR 68 and HT_{3h} infectivity, 3 x 10^8 cells/ml were infected with 50 µl of the phage suspensions. After a 30 minute incubation period with the phages, the cells were mixed with soft agar and poured onto nutrient agar plates.

3.2.6. Induction of lysis

3.2.6.1. Ultraviolet radiation (UV)

A 24 hour culture of PCB 22 grown on nutrient agar was washed and resuspended in PBS-$MgSO_4$ pH 7.2 at a cell concentration of ~10^8 cells/ml. The suspension was placed in a sterile petri dish and exposed to UV light at a wavelength of 254 nm for 5 minutes. Aliquots of 100 µl of the irradiated and non-irradiated suspensions were assayed for plaques using the double agar-overlay method described by Adams (1959).

3.2.6.2. Mitomycin C induction

PCB 22 was grown in 100 ml nutrient broth (Oxoid) in a 250ml Erlenmyer flask to an approximate density of 3 x 10^8cfu/ml. Mitomycin C (Sigma) was added to the cultures at final concentrations of 0.1, 2.5, 5 and 10 µg/ml and incubation was continued at 30°C with

shaking. The optical density of the bacterial cultures was measured at 600 nm in a Beckman DU-64 spectrophotometer at specific time intervals. The number of viable cells was determined by diluting the cells in nutrient broth, plating 0.1 ml on nutrient agar plates, and incubation at 30°C.

3.2.7. Nucleic acid Hybridisation

To determine whether the phage genome was integrated into the host chromosome, DNA from PCB 22 and PMZ 131 was isolated (Appendix B.6) and digested with the restriction enzyme, *Eco*RI, which consistently generated the same pattern of chromosomal restriction fragments. The resulting fragments were separated by agarose gel electrophoresis (Appendix B.10), transferred to positively charged nylon membrane (Hybond N^+). This membrane was probed with the single-stranded DNA isolated from the phage and labelled with ^{32}P by random priming (Appendix B.14.2) as well as a 3049 bp fragment excised from the extrachromosomal DNA using the endonuclease *Bam*HI. The latter probe was random prime labelled using digoxygenin as per the manufacturers (Boehringer Mannheim) instructions. *E. coli* chromosomal DNA digested with *Eco*RI was included as a negative control. The uncut putative RF DNA as well as *Eco*RI-digested putative RF was included as a positive control.

3.2.8. Preparation of bacteria for thin sectioning and immunogold-labelling

The 3 day incubation period required by PCB 22 for growth and production of phage prompted the investigation into the possibility of observing the development of intracellular phage. On each of 3 consecutive days, samples of PCB 22 were removed from nutrient agar plates by washing with PBS-$MgSO_4$, fixed in 2% glutaldehyde for 24 hours and prepared for thin sectioning (Appendix B.5.2). In order to positively identify the phage particles, the thin-sectioned bacteria were subjected to immunogold-labelling (Appendix B.5.3).

3.3. Results and Discussion

3.3.1. Phage detection by electron microscopy

Neither of the two *X. c.* pv. *campestris* phages resembled the novel phage with respect to morphology. Although both phages possess hexagonal heads (Figure 3.1), HT_{3h} has the long contractile tail characteristic of the family *Myoviridae*, whereas RR 68 has a short wedge-shaped tail, classifying it as a member of the family *Podoviridae*.

Electron microscopy of the supernatants obtained after the initial low speed centrifugation step of the chocolate spot isolates revealed the presence of particles which varied with respect to shape and size (Figure 3.2). This is to be expected when examining a crude sample instead of a purified sample, which would exhibit greater homogeneity in particle morphology. However, EM of these crude samples served its purpose, *i.e.* these samples resembled those obtained from PCB 22 prior to ultra- and rate zonal centrifugation, and therefore were most probably phage particles. Thus it appears that there is not a phage-free isolate of the chocolate spot pathogen that would facilitate comparative studies and serve as an indicator strain for the determination of the one step growth curve for the phage.

Only two bacteria examined, *viz. X. c.* pv. *graminis* and *X. c.* pv. *vasculorum*, were found not to be associated with phage-like particles. The supernatants of the type strain *X. c.* pv. *campestris* ATCC 528 and *X. campestris* pathovars *raphani*, *armoraciae* and *aberrans* contained particles with morphology similar to that of the novel phage (Figure 3.2). However, it was necessary to obtain additional evidence - in this case from immunoassays (section 3.3.2.) - to conclusively prove that all the particles observed by EM were the same or similar to those produced by PCB 22.

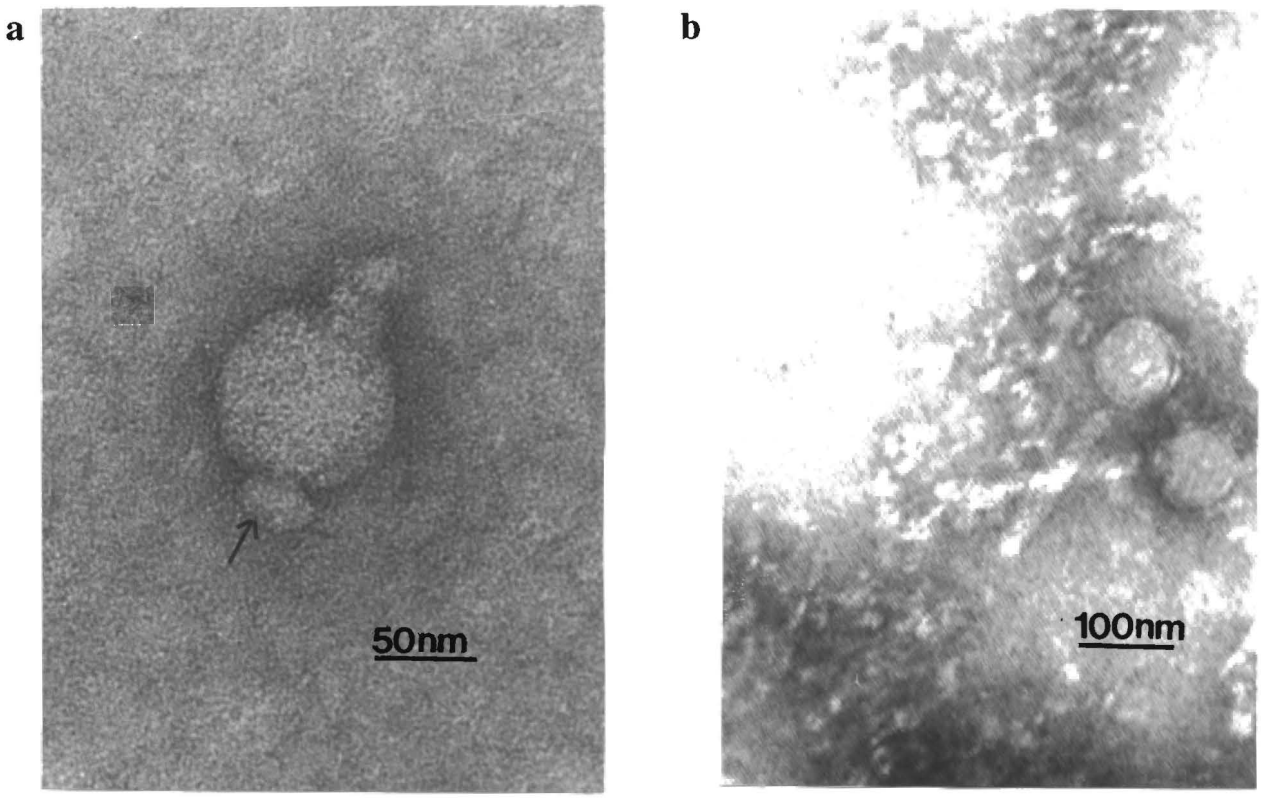


Figure 3.1. Electron microscopy of *X. c.* pv. *campestris* phages, RR 68 (a) and HT_{3h} (b) stained with 2% uranyl acetate. The arrow points to the short wedge-shaped tail of RR 68. Magnification 48 000x

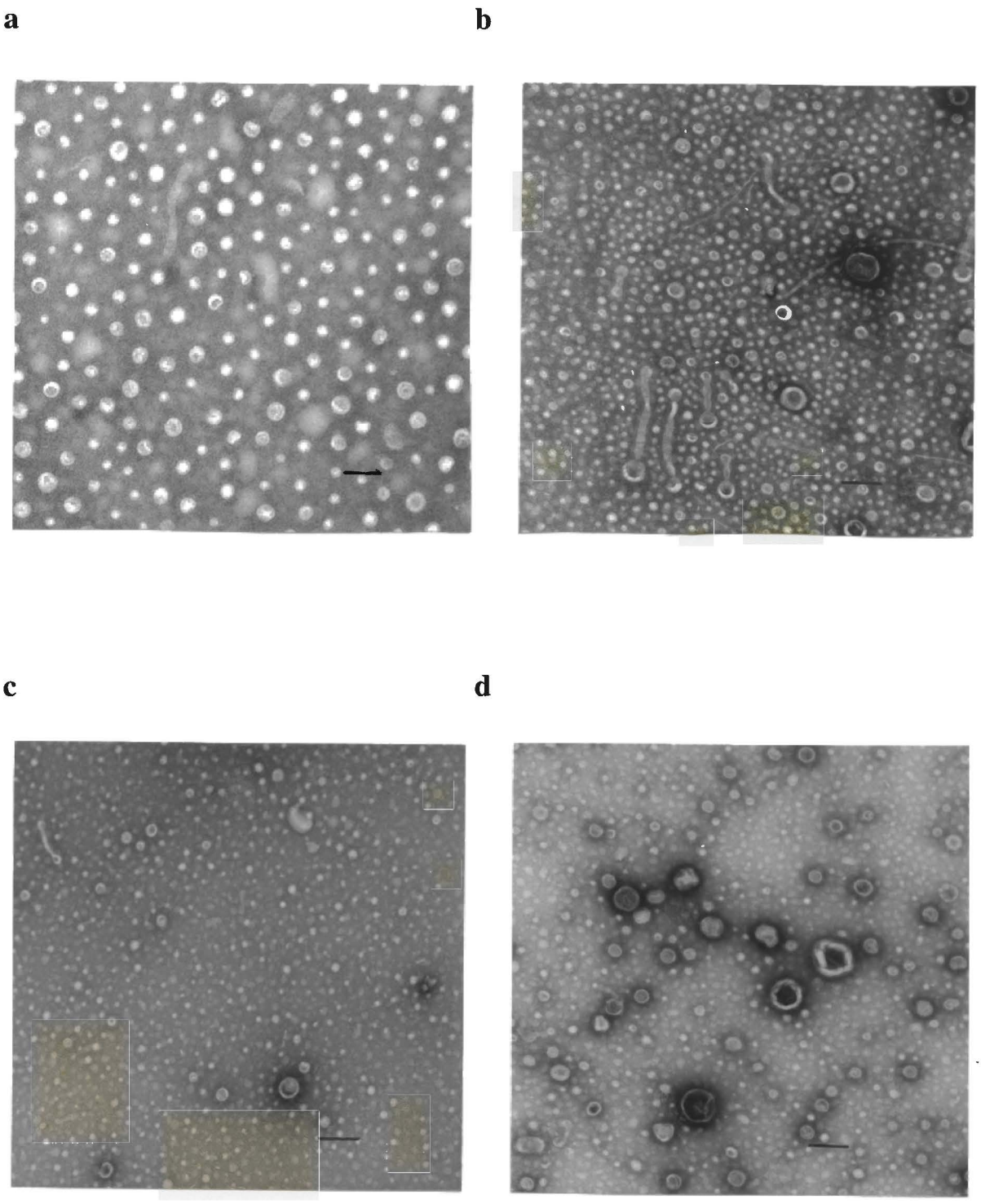


Figure 3.2. Detection of phage-like particles by electron microscopy of three representatives of the chocolate spot pathogen: PCB 21 (a), PCA 12 (b), PMZ 131 (c), and *X. c.* pv. *campestris* (d). Magnification: 30 000x. The scale bar represents 150 nm.

3.3.2. Host Range

Although a number of wide host range phages have been described for *X. campestris* (Sutton *et al.*, 1958; Alippi, 1989), many phages active against the pathovars of this species have a high degree of specificity (Liew and Alvarez, 1981a; Du Plessis *et al.*, 1981; Eisenstark and Bernstein, 1955). Host-specific phages and techniques involving serology are useful for detecting and differentiating bacterial strains of epidemiological importance. On the basis of IEB analysis, using the phage antiserum, the particles observed by EM for all the chocolate spot isolates were determined to be identical to the novel phage (Figure 3.3), thus indicating that the phage is a common feature of the chocolate spot pathogen.

IEB of *X. c.* pv. *campestris* ATCC 528 showed that the particles observed by EM are serologically identical to the phage particles produced by PCB 22 (Figure 3.3).

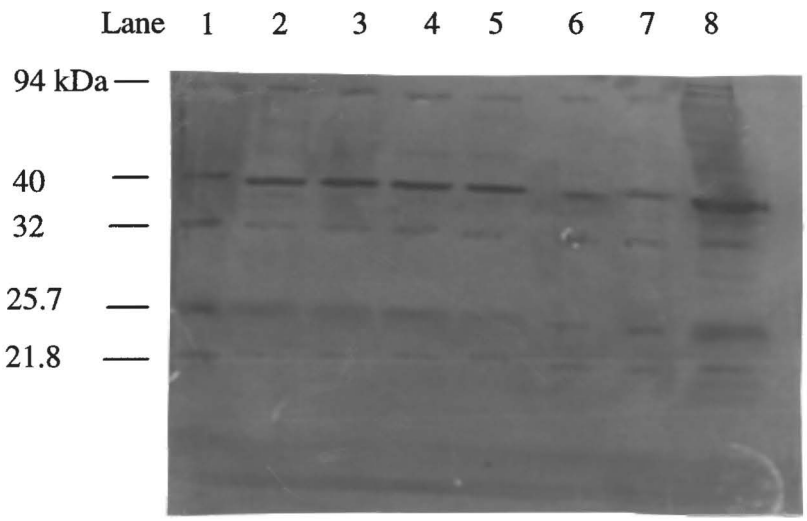


Figure 3.3. IEB analysis using the antibody raised against the purified phage. Lane 1: novel phage, 2: PCB 22, 3: PCB 32, 4: PCA 11, 5: PMZ 131, 6: PMZ133 7: *X. c.* pv. *campestris*, 8: *X. c.* pv. *raphani*

Although many phages have been described for the species *X. campestris*, phages corresponding to the morphology of this novel phage do not appear to have been identified previously, as they have not been described in literature.

A possible reason for this could be based on two facts: (i) at first glance, these particles appear to be no more than spherical bodies (ii) their presence in the bacterial cultures does not result in obvious plaque formation and appears to have no effect on the growth of the

bacteria. Therefore, if the presence of these particles was noted, it was probably ascribed to "blebbing" of the bacterial membrane, a phenomenon not uncommon in Gram negative bacteria. Furthermore, this result should not be considered too surprising, since the chocolate spot pathogen is thought to be a strain variant of *X. c.* pv. *campestris* (Kariem, 1994). What is very interesting is the fact that IEB of several other *X. campestris* pathovars, *viz. raphani*, *armoraciae* and *aberrans* produced similar results.

Phage sensitivity tests conducted to determine whether the host range of the phage extended to those xanthomonads which were found not to be associated with the phage particles, *viz.* *X. c.* pv. *campestris* ATCC 33437 and ATCC 33442, *X. c.* pv. *graminis* and *X. c.* pv. *vasculorum*, were negative. No plaques were formed on solid media, indicating that these bacteria could not serve as sensitive hosts. Neither were phage-like particles observed by EM, indicating that the bacteria remained unaffected by the attempt to infect them with the phage. From these results it is evident that the host range of the phage extends only to several pathovars of *X. campestris*.

3.3.3. Serological relationships

A phage has several antigenically distinct components. These may be capsid proteins and/or components of the tail. Certain phages cross-react serologically with one another. This is usually correlated with similarity in other properties and is used as an indication of phylogenetic relationships. Due to the unavailability of antiserum to phages RR 68 and HT_{3h}, only the antibody raised against the novel phage was used in this study.

Indirect ELISA assays, used to determine whether there was any reaction between the phage antiserum and the phages, RR 68 and HT_{3h} (Table 3.2), showed that the former phage did not react whereas HT_{3h} was half as reactive as the novel phage. This result correlated well with that obtained using IEB, which revealed that the reactive components of HT_{3h} were three proteins of molecular weights 21.8, 13.79 and 12.64 kDa (Figure 3.4b). Although not a strong reaction, the ISEM results showed the antibody to be aggregated around the phage head (Figure 3.4a). This experiment may have to be repeated in order to demonstrate the antibody reaction more clearly. However, the result obtained was interpreted as an indication that the three proteins identified by IEB are components of the phage capsid. That the 21.8 kDa protein should be a component of the phage coat is not unexpected, since the novel

phage also possesses a 21.8 kDa protein which has to be a capsid protein as this phage apparently does not possess a tail. These results suggest that the novel phage is serologically related to the *X. campestris* phage, HT_{3h}, but not phage RR 68. This is rather surprising, considering the fact that HT_{3h} belongs to the family *Myoviridae*, *i.e.* it has a hexagonal head, long contractile tail and a double-stranded DNA genome.

Table 3.2. Serological relationship between the novel phage and *Xanthomonas* phages, RR 68 and HT_{3h} as determined by indirect ELISA and IEB

Phage	Antiserum	ELISA	IEB
Novel phage	Novel phage	0.08	++
HT_{3h}	NA[a]	0.04	+
RR 68	NA	0.00	–

++ strong reaction, + partial reaction, - no reaction

[a] Not available

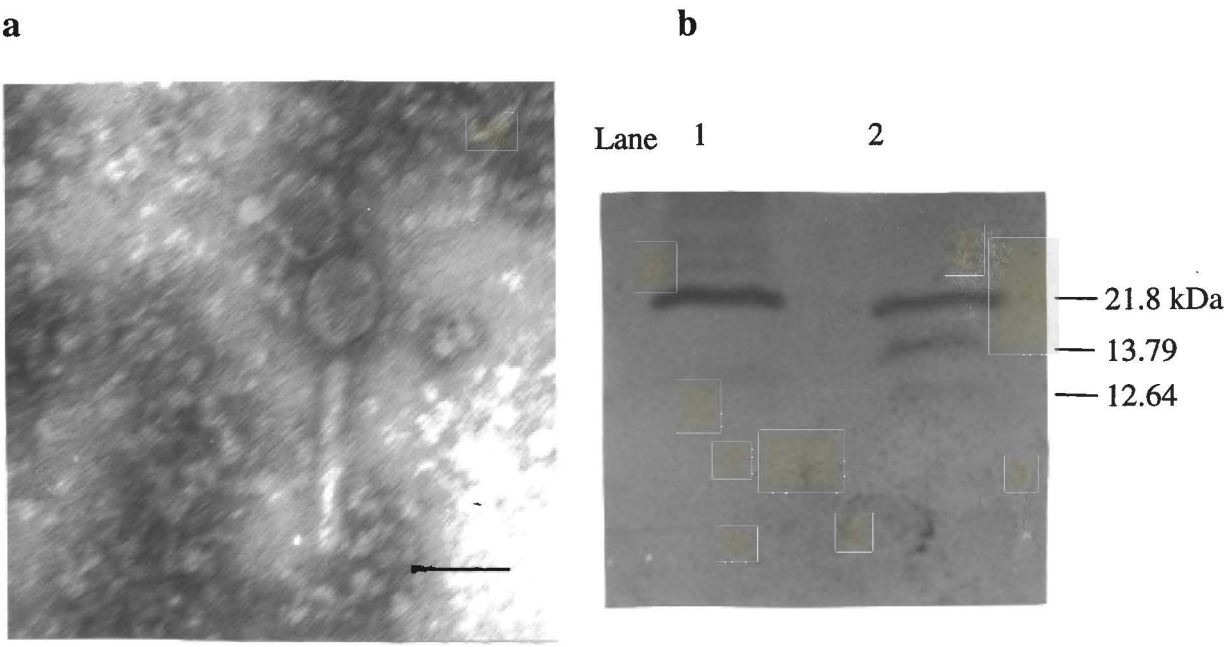


Figure 3.4. The reaction of the phage HT_{3h} with antiserum raised against the novel phage investigated by (a) ISEM; Magnification 48 000x; scale bar represents 100 nm and (b) IEB: Lane 1. Purified phage, 2. HT_{3h}

3.3.4. Evidence for the temperate nature of the phage

An established technique for discriminating between pseudolysogenic carrier strains and truly lysogenic strains, is the use of antiserum during infectivity assays. The antiserum inactivates free phage particles, thereby preventing re-infection and consequently eradicating the phage from the carrier cultures. On the other hand, bacteriophages cannot be eradicated from truly lysogenic strains in which the ability to produce phage is inherited by each successive bacterial generation.

Electron microscopy of PCB 22 grown in the presence of antiserum raised against the phage showed that free particles were still being produced after three passages in the antiserum. A similar experiment in which phage-carrier strains of *Bacteroides fragilis* were cultured in the presence of the phage antiserum demonstrated that phage production was completely eliminated after only one passage in the antiserum (Keller and Traub, 1974). The fact that phage production was not eliminated from PCB 22 after three successive subcultures in antiserum-containing media was interpreted as evidence for the temperate nature of the phage and consequently that PCB 22 was a truly lysogenic bacterium.

3.3.5. Induction of lysis

Upon infection of a suitable host, a phage may engage in either the lytic cycle which will eventually lead to lysis of the host, or the lysogenic cycle during which the phage genome is transferred from one generation to the next, in a non-infectious form (Luria and Darnell, 1967). Since the bacterial progeny retain the ability to produce phage, lysogeny is often only discovered by chance, when a small fraction of the population is spontaneously lysed, giving rise to plaques on solid media. For the novel phage, this phenomenon was observed to occur only 5 times in a period of two years during which large quantities of the bacteria were grown up regularly on a weekly basis in order to isolate the phage. Thus illustrating the rare occurrence of spontaneous lysis of the chocolate spot pathogen. The plaques observed on nutrient agar plates were turbid, as one would expect for temperate phages. Although agar plugs from these zones were removed and resuspended in PBS-$MgSO_4$ pH 7.2 to release any phages, the particles could not be used for re-infection since no indicator strain was available.

Temperate phages can be induced to enter the lytic cycle by factors such as UV radiation or mitomycin C, which either damage host DNA or inhibit DNA replication (Barksdale and Arden, 1974). However, not all temperate phages are inducible. This proved to be the situation with the novel phage. UV radiation did not result in lysis of the bacteria by the phage. Mitomycin C at concentrations of 1-10 μg/ml had a profound effect on cell numbers, with a marked decrease observed in the optical density with time (Figure 3.5). The effect of a lower concentration of mitomycin C, *viz.* 0.1 μg/ml, was only noticeable after 9 hours (Figure 3.5). Examination of the lysates by electron microscopy, revealed only isolated phage particles. These particles were too few in number to have been the cause of the observed decrease in cell numbers. The presence of these particles in the lysate may be attributed to phage production prior to the addition of mitomycin C.

Since lysis of the bacteria could not have been due to the induction of the phage, the decrease in bacterial numbers may be the result of the production of a bacteriocidal compound, *e.g.* a bacteriocin. Bacteriocins are lethal compounds which occur in the form of low molecular weight proteins or they may be particulate, resembling phage tails or empty phage heads attached to tails (Bradley, 1967).

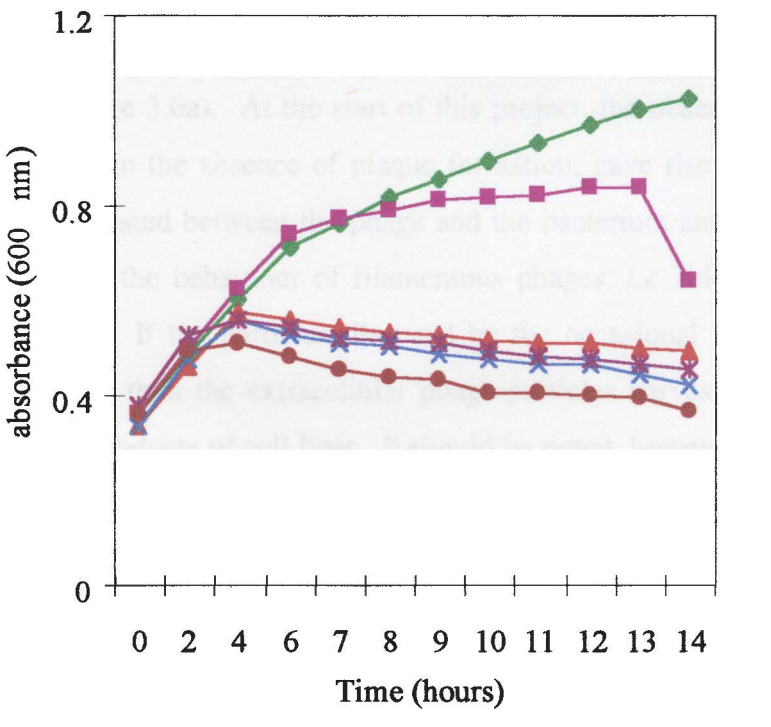


Figure 3.5. Effect of mitomycin C on the growth of PCB 22

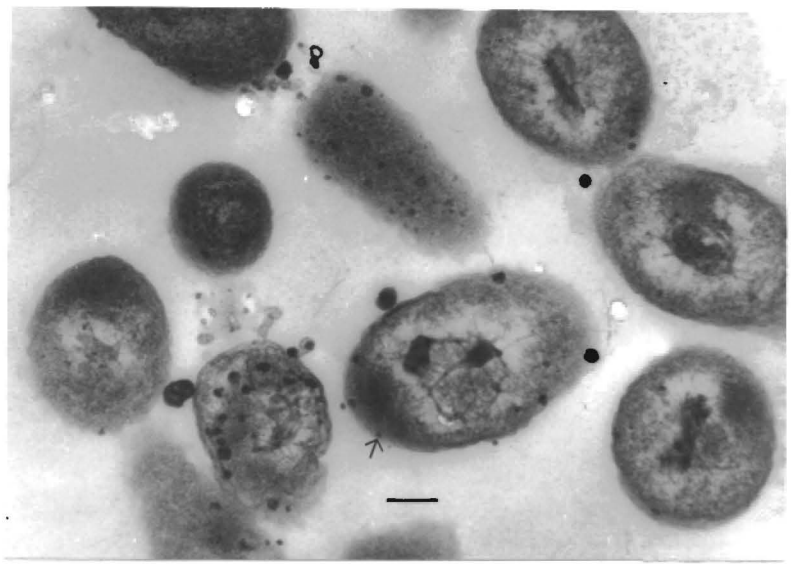
Studies on bacteriocin production by xanthomonads are limited, with only *X. c.* pv. *glycines* having been conclusively proven to produce bacteriocins (Fett *et al.*, 1987). Other plant pathogens capable of bacteriocin production include members of the genera *Erwinia*, *Pseudomonas* and *Corynebacteria* (Vidaver, 1976). Like temperate phages, bacteriocins can be induced, resulting in bacterial lysis. As no unusual structures were resolved by electron microscopy, it is likely that the bacteriocidal effect was caused by a non-particulate molecule. However, more data is required before it can be said with any certainty that the chocolate spot pathogen is capable of producing a bacteriocin.

3.3.6. Cytology

Having noticed that the phage yield was highest after 3 days of incubation at 30°C, it was decided to conduct an electron microscopic study, using thin sectioning and negative staining techniques, to investigate the stages in the life cycle of the phage. By studying thin sections of infected bacteria it is possible to observe the intracellular development of phages, leading to eventual lysis. This method has proved invaluable in the study of the lipid-containing phage ϕ6 (Bamford *et al.*, 1976) and phage T4 (Black *et al.*, 1994).

Thin sections of PCB 22, prepared after 1, 2 and 3 days of growth, showed no visible differences in the intracellular environment of the bacteria. No more than 1 or 2 particles approximating the size of the phage, were seen inside the cells over the 3 day period. The few particles observed were mostly located towards the outer regions of the cell, close to the cell wall (Figure 3.6a). At the start of this project, the observation that extracellular phage was produced in the absence of plaque formation, gave rise to the theory that a symbiotic relationship existed between the phage and the bacterium and that the release of this phage might emulate the behaviour of filamentous phages, *i.e.* release via extrusion through the host cell wall. If the particles liberated by the occasional bursting cell (Figure 3.6b) are indeed phages, then the extracellular phage particles harvested for analytical purposes are probably the products of cell lysis. It should be noted, however, that the observed disruption of the cell wall may be a result of the sample preparation technique for electron microscopy and not due to cell lysis. The absence of plaques can be explained by the assumption that the rate of lysis is lower than, or in equilibrium with bacterial growth, thus giving the illusion of confluent growth on solid media.

a



b

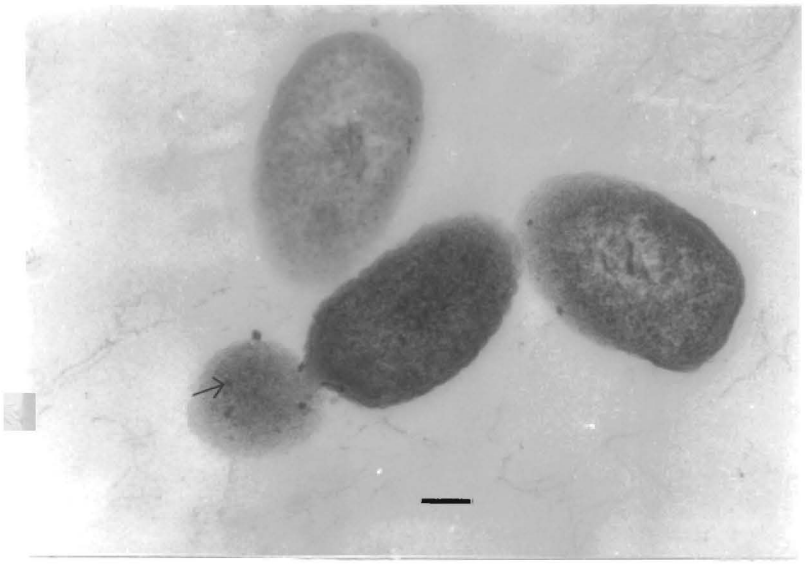


Figure 3.6. Electron micrographs of the negatively stained, ultrathin-sectioned PCB 22 grown on nutrient agar for 3 days. (a) internal phage-like particles, (b) apparent cell lysis releasing the particles from the bacterium. The arrows point to the phage-size particles. Magnification 30 000x. The scale bar represents 150 nm.

Unfortunately, immunogold labelling could not confirm that the phage-sized particles were indeed phages since these structures were not often detected in the cells.

On the rare occasion when gold particles were observed on the grids, they appeared to be clustered around entities not clearly visible within the bacteria. Although these may have been phage proteins, this in no way elucidated the nature of the phage-sized particles occasionally observed.

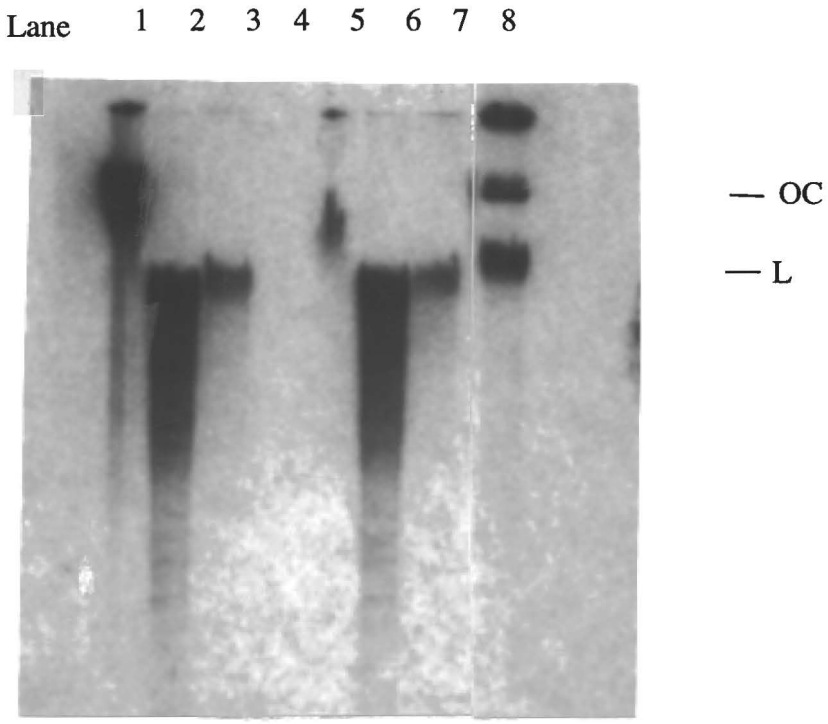
3.3.7. Nucleic acid analysis

Previous research has shown that the temperate bacteriophage genome exists persistently in host cells either as an episome, *e.g.* filamentous phages (Marvin and Hohn, 1967) and phage P1 (Prentki *et al.*, 1977), or as a prophage integrated into the host chromosome, *e.g.* bacteriophage λ (Weisberg and Landry, 1983). Until the discovery of integration by two single-stranded filamentous phages infecting *Xanthomonas* (Dai *et al.*, 1987; Kuo *et al.*, 1987), it had always been thought that integration was the exclusive property of double-stranded DNA phages. This phenomenon, termed neolysogenization, has since been described for ϕXo, ϕXv and VSK, filamentous phages which infect *Xanthomonas* and *Vibrio cholera* O139 respectively (Lin *et al.*, 1994; Kar *et al.*, 1996). This indicates that neolysogeny may be a widespread phenomenon and not just confined to a few of the phages infecting *Xanthomonas*.

Since the novel phage was found to contain a single-stranded DNA genome, Southern hybridisation was used to determine whether the phage genome existed solely as an episome or whether it was capable of neolysogenization. Total DNA extracted from the host, PCB 22, was first probed with the ^{32}P-labelled mature phage genome. As expected, the probe hybridised strongly with the linear and open circular forms of the putative RF DNA (Figure 3.7, lane 8). Hybridisation between the single-stranded phage DNA and the PCB 22 and PMZ 131 chromosomal DNA is also evident (Figure 3.7a). However, no conclusions concerning phage genome integration could be made based on the banding pattern of the *Eco*RI-digested bacterial total DNA sample. The complex banding pattern was attributed to the use of the entire phage genome as a probe. Consequently, the *Eco*RI-digested PCB 22 DNA was probed with a 3049 bp fragment excised from the putative RF of the phage

genome. This probe hybridised to two bands of 12.7 kb and 9.9 kb in both the *Eco*RI-digested extrachromosomal and PCB 22 DNA (Figure 3.7b).

a



b

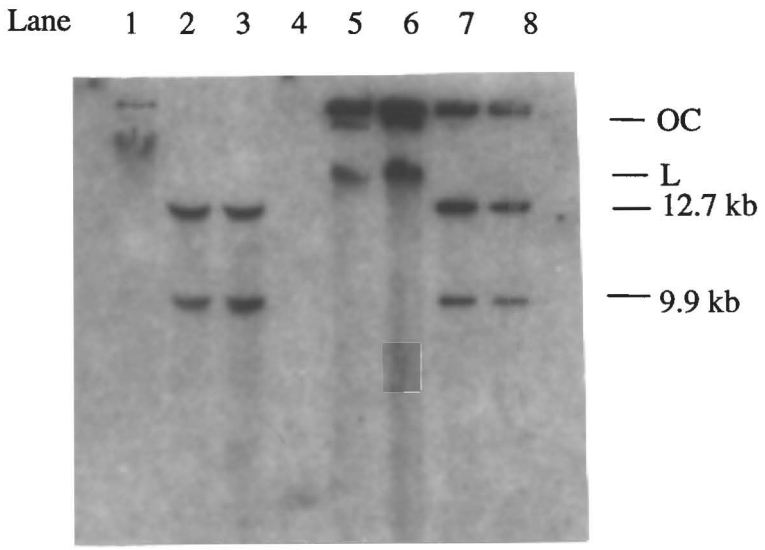


Figure 3.7. Southern hybridisation of genomic DNA from the phage host PCB 22 to (a) the ^{32}P-labelled mature phage genome. Lane: (1) uncut PCB 22 genomic DNA (2) *Eco*RI-digested PCB 22 genomic DNA (3) *Hin*dIII-digested PCB 22 (4) *E.coli* genomic DNA (5) uncut PMZ 131 (6) *Eco*RI-digested PMZ 131 (7) *Hin*dIII-digested PMZ 131 (8) uncut phage RF; and (b) to the digoxygenin-labelled 3049 bp putative RF fragment . Lane (1) uncut PCB 22 genomic DNA (2 & 3) *Eco*RI-digested PCB 22 genomic DNA (4) *E.coli* genomic DNA (5 & 6) uncut phage RF (7 & 8) *Eco*RI-digested RF . L : linear, OC: open circular

Due to the non-phage-like nature of the 3049 bp probe, elucidated by sequence analysis (see chapter 4), the result shows only that the DNA used as a probe is present on the extrachromosomal element and does not confirm the integration of the phage genome into either this element or the bacterial chromosome. Since the size of the extrachromosomal element precludes it from being the replicative form of the single-stranded phage genome, hybridisation of the ssDNA probe to this DNA element was interpreted as evidence for the integration of the actual phage RF into the extrachromosomal DNA element.

Ideally, integration would have been investigated by hybridisation of the single-stranded genome to the restriction enzyme-digested extrachromosomal DNA in order to localise the site of integration. This experiment would also have facilitated the cloning of the actual phage genome, and thus furthered the molecular characterisation of the phage. Thus, in addition to its combination of morphology and nucleic acid type, the phage shown to be of a temperate and non-inducible nature has two further very interesting features: firstly, the slight serological similarity between this phage and the *Xanthomonas campestris* phage HT_{3h}; secondly, the apparent integration of the phage genome into an extrachromosomal element.

CHAPTER 4

The Molecular Cloning and Sequencing of the Extrachromosomal Element

Contents

The Molecular cloning and sequencing of the extrachromosomal element

Summary

The 53.9 kb extrachromosomal element was cloned and several recombinant plasmids sequenced. No typical phage-like genes were identified. The subcloning of a 3049 bp fragment generated by a *Bam*HI restriction endonuclease digest of the clone pS32.1 yielded plasmid pSSI which upon subcloning of a 1523 bp *Bst*XI generated fragment yielded pSBXA3. The plasmids pSSPT2 and pSSPL3 were generated by a *Pst*I restriction enzyme digest of pSSI. Sequencing of all these clones resulted in the identification of 201-262 bases which shared approximately 98% similarity at the nucleotide level to the 3' end of a group of *Xanthomonas* avirulence (*avr*) genes, 141-311 bases with a high degree of nucleotide similarity to the 5' of the *avr* genes. These two regions of *avr* gene similarity, are interrupted by 1259 bp sharing 99% nucleotide identity with the 3' region of the 6938 bp *X. campestris* transposon, ISXC5 and 298 bases with 97% similarity to the 3' end of the transposon. The 1259 base and 298 base regions are in turn separated by an unsequenced region of 518 bases.

4.1. Introduction

There is no doubt about the great diversity of bacteriophages in nature. To date, at least 4500 different bacteriophages - doubtless only a small fraction of existing bacterial viruses - have been described in literature (Ackerman, 1996). The phages are largely classified on the basis of gross morphology and the nature of their nucleic acid. Since morphological similarities do not necessarily imply evolutionary relatedness, analysis of the phage nucleic acid is of prime importance in the determination of phylogenetic relationships as well as in phage taxonomy.

Evidence of the complexity of this group of microorganisms is provided by the genomic diversity which exists within a single well-defined phage family (Monod *et al.*, 1997; Repoila *et al.*, 1994). Unlike animal viruses, no lines of descent based on the gradual accumulation of point mutations have been established for bacterial viruses (Casjens *et al.*, 1992). Botsteins' (1980) modular theory of phage evolution provides a plausible explanation for the genetic variability of bacteriophages. According to this theory, natural selection acts not at the level of the individual phage particles, but at the level of individual genetic segments (modules) that are interchangeable by recombination. Thus the phages found in nature represent favourable permutations and combinations among compatible modules. This theory of phage evolution has gained popularity due to its success in explaining the genetic variability of the lambdoid (Casjens *et al.*, 1992) and T4 phages (Monod *et al.*, 1997; Repoila *et al.*, 1994).

In the case of the phage isolated from PCB 22, neither morphological analysis nor determination of the type of nucleic acid facilitated classification of the phage into one of the accepted families of phages. Since the mature single-stranded (ss)DNA hybridised strongly with the extrachromosomal DNA isolated from PCB 22 (chapter 3), it was thought that this double-stranded (ds)DNA was either the RF of the ssDNA isolated from the mature particles, or that the phage genome had integrated into this element. Therefore, to determine the nature of the extrachromosomal DNA and to hopefully gain insight into the genome of the phage, cloning and sequencing of the dsDNA was undertaken in order to locate any phage-like genes. Location of these genes would also serve as further proof that the particles released by PCB 22 are indeed phages, since the conventional method using infectivity assays did not work in this case due to the lack of a sensitive indicator strain (chapter 3).

4.2. Materials and Methods

4.2.1. Bacterial strains, plasmids and media

The chocolate spot isolate, PCB 22, was routinely grown on nutrient agar (NA) (Biolab). *E. coli* JM109 (Dept. Culture collection) was cultivated in nutrient broth (Oxoid) or on NA, and used for all DNA manipulations. Bluescript pSK was used as the cloning vector and was introduced into *E. coli* JM109 by transformation.

4.2.2. Restriction enzyme digestion and cloning of the RF DNA

Putative RF DNA was isolated from PCB 22 as described in Appendix B.5., following the growth of PCB 22 for 48 hours on nutrient agar (Biolab) and a low speed centrifugation step to pellet the bacterial cells (Section 2.2.4.5b). The putative RF DNA was digested with a number of enzymes (Section 2.3.4.) and based on the restriction patterns, the restriction endonuclease *Bam*HI was selected for use in the cloning of this DNA, as the enzyme produced a large number of fragments within the size range of 9.5-2.4 kb. A detailed account of the cloning protocol has been included in Appendix B.9.-B.13. Briefly, following restriction endonuclease digestion with *Bam*HI, the fragments were separated on a TAE gel, collectively excised and purified using the Geneclean (Bio101) kit. This DNA was ligated into the *Bam*HI site of pSK - from which the 5′-phosphate group had been removed – using the Fastlink ligation kit (Boehringer & Mannheim). Recombinant plasmids were used to transform competent *E. coli* JM109 cells by heat shock. Several of the resultant clones were selected for end-sequencing.

4.2.3. Subcloning

Based on the results obtained from end-sequencing two of the clones, pS43.1 and pS32.1, the latter clone was selected for further analysis. A partial restriction map of pS32.1 was constructed in order to locate restriction enzyme sites for subcloning experiments. Digestion of pS32.1 with *Bam*HI revealed two fragments of 4946 and 3049 bp in size, suggesting that a chimera had formed during the original ligation reaction. The two fragments of 3049 bp and 4946 bp were subsequently cloned into pSK separately, to form pSSI and pSB18 respectively. pSSI was digested with restriction endonucleases *Bst*XI and *Pst*I, releasing fragments of 1523 bp and 500 bp in size. The former fragment was ligated into the *Eco*RV site of pSK following the production of blunt ends, giving rise to the recombinant plasmid

pSBXA3. The vector and remaining insert following *Pst*I-digestion was religated to form pSSPT2.

4.2.4. Nucleotide sequencing and analysis

The DNA used in sequencing reactions was purified by CsCl-ethidium bromide density gradient centrifugation (Appendix B.7.). The nucleotide sequence was determined by the dideoxy chain termination method of Sanger *et al.* (1977). The thermosequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP from Amersham Pharmacia Biotech UK Ltd was used as per the manufacturer's instructions. Used in each reaction was 20-48 ng of DNA and 1 µl of 2 pmol/µl Cy5 labelled fluorescent primers:

Forward 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′

Reverse 5′-GAGCGGATAACAATTTCACACAGG-3′

These were obtained from the Synthetic DNA Laboratory, Dept. of Biochemistry, UCT. Sequence reactions were run on an ALFexpress automated DNA sequencer from Pharmacia Biotech, Uppsala, Sweden using 0.5 mm spacers and run as per manufacturer's instructions. Gels (5% w/v) were poured using Long Ranger gel solution from FMC Products, Rockland, Maine U.S.A.

Computer analysis of DNA sequences was performed using the Wisconsin Package, version 9.1 (Genetics Computer Group, Madison Wisconsin). Homology searches were performed using the new BLAST package at the National Centre for Biotechnology Information via the Internet (www.ncbi.nlm.nih.gov).

4.3 Results and discussion

4.3.1. Cloning

A number of the recombinant plasmids obtained following the cloning of the *Bam*HI-digested extrachromosomal DNA, contained inserts with more than one *Bam*HI site (Figure 4.1). Based on the analysis of the end-sequencing results for one of these clones, pS32.1, which contained 4946 bp and 3049 bp *Bam*HI-released fragments, it was decided to sequence as much (if not all) of the insert DNA. Initially, this was to be achieved using the Heinekoff shortening protocol (Heinekoff, 1984) to generate a range of overlapping deletions which could then be used for nucleotide sequencing. Although every aspect of the experiment was investigated a number of times using the appropriate controls, none of the

"shortened" plasmids could be used to transform competent *E. coli* JM109 cells. As a result, a partial restriction map of pS32.1 was constructed (Figure 4.2). This facilitated the design of a subcloning strategy (Figure 4.2) that would allow a larger region of the pS32.1 insert to be sequenced. Two of the endonucleases used in the subcloning of the 3049 bp insert, *Bst*XI and *Pst*I, yielded only partial success in the goal to sequence this fragment completely. Religation of the vector and remaining insert following digestion with *Bst*XI was not achieved despite many attempts. Consequently, the sequence obtained is not contiguous.

4.3.2. Nucleotide sequence analysis

4.3.2.1. Identification of a sequence involved in bacterial pathogenicity

The results for end-sequencing of two clones, pS43.1 and pS32.1 (obtained after ligation of the *Bam*HI-digested extrachromosomal DNA into pSK) were not encouraging with respect to identifying phage-specific sequences. Instead, pS43.1 sequenced in one direction only did not show significant similarity to any nucleotide sequences in the databases (Figure 4.3 and Table 4.1.). Analysis of the end sequence obtained from pS32.1 revealed a 201-262 base region sharing approximately 98% similarity to the 3' end of a group of *Xanthomonas* avirulence (*avr*) genes, while 147-231 bases shared a high degree of similarity with the 5' end of the avirulence genes (Figure 4.4, Table 4.2).

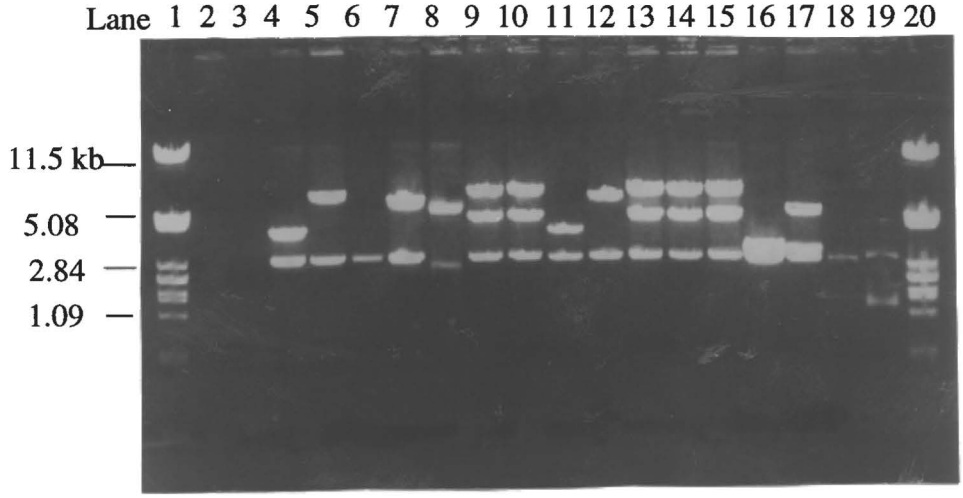


Figure 4.1. Recombinant plasmids digested with *Bam*HI. Lane 1 and 20: molecular weight marker, λ-*Pst*I

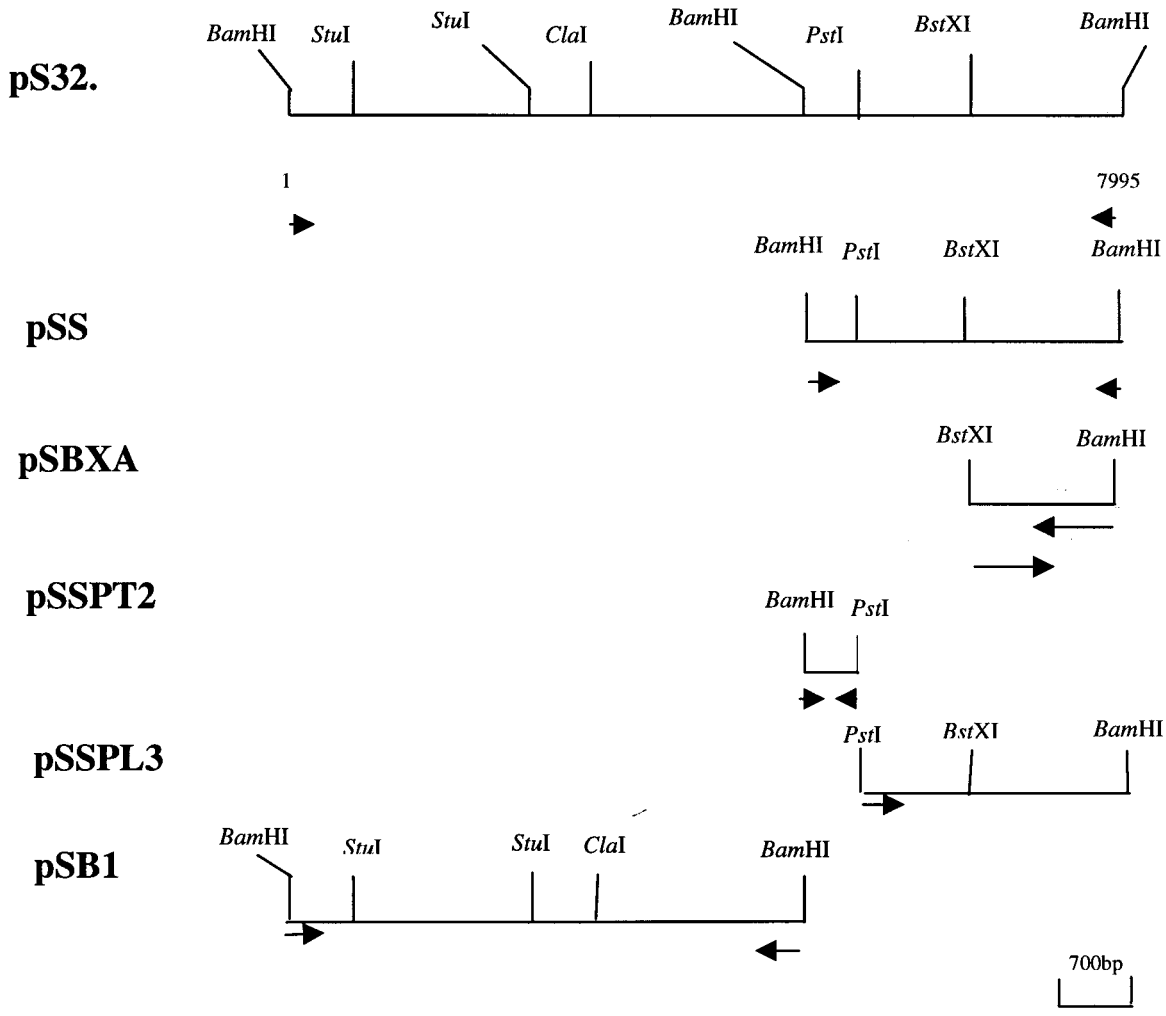


Figure 4.2. Partial restriction map of plasmid pS32.1 and subcloning strategy. The arrows indicate the direction of sequencing

This unexpected but interesting result prompted further subcloning of pS32.1. End sequencing of the 2 subclones pSB18 and pSS1 showed that the former sequence in the reverse direction did not share similarity to nucleotide sequences currently in the databases. While the pSB18 sequence in the forward direction is identical to the pSS1 reverse sequence in the region homologous to the *avr* gene, this homology does not extend to the full length of the sequences obtained, *i.e.* the pSB18 forward sequence has a 54% similarity to the pSS1 reverse sequence (Figure 4.5). In addition, these two regions differ with respect to endonuclease restriction sites, further indicating that the 3049 bp insert of pSS1 is not a smaller fragment of the pSB18 4946 bp insert.

The *avr* genes with which the extrachromosomal DNA sequence shares identity (Table 4.2) all belong to a major family of homologous *avr*/pathogenicity genes widespread in the genus *Xanthomonas*. The most striking feature of this gene family is the presence of nearly

identical 102 bp tandem repeats in the central region within the genes which appear to determine the phenotype exhibited towards plants (Herbers *et al.* 1992; Yang *et al.* 1994; Hopkins *et al.* 1992; De Feyter *et al.* 1993). These genes may be plasmid-borne or located on the chromosome (for reviews see Vivian and Gibbon, 1997; Swings and Civerolo, 1990).

Avirulence (*avr*) genes present in phytopathogenic bacteria interact with complementary resistance genes in the plant. This triggers the hypersensitive response (HR) resulting in rapid, localised necrosis of plant tissue, thereby preventing the spread of the pathogen and onset of disease (resistant response as a result of an "incompatible" reaction). In the absence of either the *avr* gene or the matching plant gene, the plant fails to recognise the bacterium as a pathogen, usually resulting in disease (susceptible response as a consequence of a "compatible" reaction) (Staskawicz *et al.* 1995; see reviews by Keen, 1990, Vivian and Gibbon, 1997). Since incompatible reactions effectively restrict the host ranges of pathogens, *avr* genes are considered to be negative factors, disadvantageous to the bacterium. Obviously the *avr* genes must have functions beneficial to the bacterium which outweigh the negative effects. However, in most cases these "beneficial" functions remain unknown although several a*vr* genes have been found to confer a selective advantage on the bacteria with respect to fitness and/or symptom formation (Dangl, 1994; Kearney and Staskawicz, 1990).

4.3.2.2. Identification of a DNA region homologous to a *Xanthomonas* transposon

Transposable elements were initially identified during studies of model genetic systems as discrete mobile sequences capable of generating mutations as a result of insertional inactivation of genes (Jordan *et al.* 1968). They have subsequently been shown to play an important role in the dissemination of antibiotic resistance genes amongst bacteria, chromosomal deletions, inversions, episome integration/excision as well as (in)activation of genes (reviewed by Kleckner, 1981; Louarn *et al.* 1985). In recent years, insertion sequences have also become associated with pathogenic and virulence functions of plant pathogens, *e.g.* *Agrobacterium* (Otten *et al.* 1992).

Computer analysis of the pSS1 reverse sequence revealed that the region sharing homology with the 3' end of *Xanthomonas avr* genes was interrupted by approximately 430 nucleotides

```
  1  CCGACAGCGT GCTGCGCGCG CGGCTGGATT CGATGTTCAT GGAGCACGGC
 51  GTGCAGACCC CGACCAATGC GATCGANACC TCGTCGCTGC CGGTGACCTC
101  CACCTTGTTG CGCGGCAGCG ACATGCTGAC CGCGCTGCCG GTGGAGTCGG
151  TGGCGCCGCT GATCCAGGCC AAGTTGCTCA CCGTGCTGCC GATCGAATTG
201  GGCGTGCGGA TGGAGTCGTT CGGCATCATC CGTCGGCGCG ATTACATCCT
251  GCCGCCCGGC GCCGAGCGCA TTTTGCAGGC CCTGCGCAGC ACCGCACGTC
301  GCCTGTACCC AGGCCTGCGC CANCCCGGNA TCGCCTGTCT AAGCAGGCTC
351  NTCCATTTCC ATTCCTTTTC GGCATACCAG CCTGTGATTT TTTCATTGGC
401  TGGCGCTAGA CCGGGCGCCT ATACCTGTGC GTCAGATCGC ACGCTGAAGC
451  GCACGTGAAG TCGGGCAAGT NATCBGGCGG CCAAGCCGCC ATGTAGTGGG
501  AAGCGGAACC GGGCCCCAGT GCCCAGGTCC CATCGATCAC GCGAGCCCGT
551  TGGTCGCGAC AACCAGTCTT CAGTCGTCTT GCGTCCTGGG AGGGATTCGG
601  ATGTACACAT TTTCCAGCCA TGCTTCGGCA GGCACGGGCN CGCGTGCNCT
651  C
```

Figure 4.3. Partial nucleotide sequence of pS43.1 obtained by end-sequencing the clone in the forward direction.

Table 4.1. Similarity of the indicated sequences to pS43.1

Sequences producing significant alignments	Identical/aligned	E Value
E. coli K-12 chromosomal region from 67.4 to 76.0 minutes	22/22	0.024
E.coli K-12 MG1655 section 303 of 400 of the complete genome	22/22	0.024
Chlamydomonas reinhardtii cell wall lytic enzyme mRNA	19/19	1.5
C. reinhardtii mRNA for gamete lytic enzyme (GLE)	19/19	1.5

Note: The second column gives the number of identical nucleotides for the two sequences compared over the indicated length of computer-aligned nucleotides. The third column gives the probability derived from the BLASTN score for obtaining a match by chance.

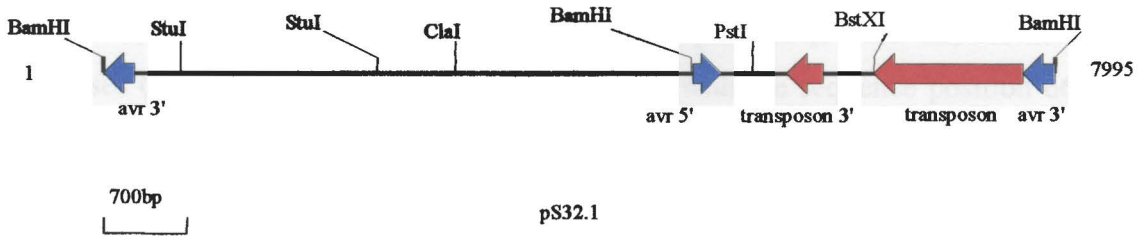


Figure 4.4. Regions of pS32.1 which share nucleotide sequence identity with *Xanthomonas* avirulence (*avr*) genes and the transposon ISXC5. The arrows indicate the length and direction of the sequence.

Table 4.2. Similarity of DNA sequence from the extrachromosomal element to sequences in the database

Similarity	Region of similarity[a]	% Similarity[b]
X. campestris PthN gene	3339-3578	97
	163-391	95
X. c. pv. *malvacearum* avrb6	3502-3756	96
	342-570	95
X. campestris avrBs3	3965-4219	96
	376-604	91
X. campestris avrBs3-2	3658-3895	96
	100-302	94
X. oryzae avrXa10	3382-3583	94
	30-207	93
X. campestris avrBsP 5' end	1-147	93
X. campestris transposon ISXc5	4864-6126	99
	6644-6938	97

[a] the regions of similarity correspond to those illustrated in Figure 4.4.

[b] percentage similarity at the nucleotide level to the cloned regions of the extrachromosomal DNA illustrated in Figures 4.2 and 4.4 and the nucleotide sequence in Figure 4.6.

which showed 100% homology to a 6.938 kb *X. c.* pv. *citri* transposon, ISXC5. The complete sequencing of the 1.523 kb insert (containing the aforementioned sequence) within the subclone pSBXA3 (Figure 4.2) showed that 1.258 kb (Figure 4.6) shared a very high degree of homology to the 3' region of ISXC5 extending from nucleotide position 4864 to 6126 (Table 4.1 and Figure 4.3). Sequencing of pSSPL3 (Figure 4.2) revealed a further 298 bases (Figure 4.6) which share a 97% identity with the sequence position 6644 to 6938 of ISXC5 which contains the 40 nucleotides repeated at both the 5' and 3' ends of the transposon. Additional sequencing (prevented by the only partial success of the subcloning strategy) of the original 3049 bp insert of subclone pSS1 may reveal further homology to the 3' end of ISXC5.

```
1     GGATCCTGGTACGCCCACGGCTGCCGACCTGGCAGCGTCCAGCACCGTGATGCGGGAACA
      ************************************************************
70    GGATCCTGGTACGCCCACGGCTGCCGACCTGGCAGCGTCCAGCACCGTGATGCGGGAACA

61    AGATGAGGACCCCTTCGCAGGGGCAGCGGATGATTTCCCGGCATTCAACGAAGAGGAGCT
      ************************************************************
130   AGATGAGGACCCCTTCGCAGGGGCAGCGGATGATTTCCCGGCATTCAACGAAGAGGAGCT

121   CGCATGGTTGATGGAGCTATTGCCTCAGTGAGGCTCAGTCGGT.GACTACCTGATACGCC
      ****************************************  ***** ****    *
190   CGCATGGTTGATGGAGCTATTGCCTCAGTGAGGCTCAGTCGGGGGACTATCTGANGGTCG

180   C.........................................................

250   GCAGGGATTGGTGTAAGAAACCTTTACTTACAGCAAGTTAGCTCACTTTTGGCTGTTTTT

181   ..................................................AGGGTTACGT
                                                       *****
310   TACACGAATCCCTGCCGACCCTCATGCAGCACCGGGCTTGGCACGGCGGTAGGGTCTTCC

191   AGACACTCAAGACCCTCGTTGCGCGTASCCGGTTCCGAACTCTACAGGCGATAGGTCGCC
      *** *                 ***   * *** *      **   * *     *
370   AGAAAGGTGCCGAGAAACCGCGCCGTGGTCAGTTGCAGCGCATAACCGAGGCGACTGCTG

251   AGTTGAACCGTGACGGCGTTTTGGGTTGTAGAACATCTCGATATAGTCGAATA.......
          *   * **  **** *             *          *       ****
430   TCGCGTCGCTTGGTGGCGATCCACTCGCGGTCATCGTCGTCCAGGTGGAAATAGCGCGCC

304   .........................................................

490   AGCTCTTTCGCTGGAAAGCGTGTCTGGATAGCGGCCGTAGCGTTCCCGTTGTGTGGTGGA

304   ...........................CCTCGGCGCGTGCGGCGTCCTTTGTGGGAT
                                 *********    ** * *    ***
550   CAGGAAGCTGACCGGCATGGCGTCAAAGCGCCTCGGCGCAGAAGGTGACTGGCGTGACGA

334   AGGCCCGTCGCCTGATACGCTCGCGTTTGAGCAGACCGAAGAAGCTCTCCACG.......
       *  *   ** *  ** *   ******  ***** **   *  **  **  *
610   TGCGCGTGCGGCCAATGCTACCGCGTTGCAGCAGCCCAGCGCGGCGGTCGCCAGTCACAA

387   .........................................................

670   GGTAATCGGCCTCGCTGGCCAGTGCCATCGCCAGCAGGGAACGCATCGTTCGGATCATTG

387   ..........................................GGTGCGTTGTCGTGGC
                                                ** *** **
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730   ACCTCGATGCGATCAGGCAGAGGCGGCAATACATGCAGCACAACGGSGCGCTGCATGTTG

403   AGTTGCCACGCCGACTCATKGCTGGCACACCAAGCCATGGGACGCCAGGAAACTGCGCCA
          *  **       *        **   *                 *   *       *
790   TTGATCANCGTGCCGACGCGATGCGCGGGCAGGATTGCCTTGATCTTCGGGTAACGGSTC

463   GTCATCGCTGGTGTAGACAGACCCTTGGTCCGAGTGAACCAAGCAACCAGCGTTGGGTTT
           *   **  *   **   *   ****  ** * *    ***   * * **
850   ACGNGGGGCAGTTCATCAAGCTGCGCCGTCCCTGTCACCAGTTCAAAGCGNGNTGCAAVC

523   GCGCCGCCACACCGCAGACAACAAGGGCTGCMCGACCAAC
         **** * *    *         ****    * *****
910   CAACCGCGANAAATGATNTCGGGNGGGCYTTNCNACCAAA
```

Figure 4.5. Nucleotide sequence alignment of pSB18 and pSS1. upper line: pSB18 from 1 to 562 lower line: pSS1, from 70 to 949. Homology: 54%. * indicates identical nucleotides. Gaps introduced to optimise the alignment are indicated by dots.

```
   1  GGATCCTGGT ACGCCCACGG CTTGCCGACC TGGCAGCGTC CAGCACCGTG
  51  ATGCGGGAAC AAGATGAGGA CCCCTTCGCA GGGGCAGCGG ATGATTTCCC
 101  GGCATTCAAC GAAGAGGAGC TCGCATGGTT GATGGAGCTA TTGCCTCAGT
 151  GAGGCTCAGT CGGGGGACTA TCTGAGGGTC GGCAGGGATT GGTGTAAGAA
 201  ACCTTTACTT ACAGCAAGTT AGCTCACTTT TGGCTGTTTT TTACACGAAT
 251  CCCTGCCGAC CCTCATGCAG CACCGGGCTT GGCACGGCGG TAGGGTCTTC
 301  CAGAAAGGTG CCGAGAAACC GCGCCGTGGT CAGTTGCAGC GCATAACCGA
 351  GGCGACTGCT GTCGCGTCGC TTGGTGGCGA TCCACTCGCG GTCATCGTCG
 401  TCCAGGTGGA AATAGCGCGC CAGCTCTTCG CTGGAAAGCG TGTCTGGATA
 451  GCGGCCGTAG CGTTCCCGTT GTGTGGTGGA CAGGAAGCTG ACCGGCATGG
 501  CGTCAAAGCG CCTCGGCGCA GAAGGTGACT GGCGTGACGA TGCGCGTGCG
 551  GCCAATGCTA CCGCGTTGCA GCAGCCCAGC GCGGCGGTCG CCAGTCACAA
 601  GGTAATCGGC CTCGCTGGCC AGTGCCATCG CCAGCAGGAA CGCATCGTTC
 651  GGATCATTGA CCTCGATGCG ATCAGGCAGA GGCGGCAATA CATGCAGCAC
 701  AACGGCGCGC TGCATGTTGT TGATCATCGT GCCGACGCGA TGCGCGGGCA
 751  GGATTGCCTT GATCTTCGGG TAACGGCTCA CGGGCGCAGT TCATCAAGGT
 801  GGCCGTCCCT GTCACCAGTT CAAAGCGTGC TGCAAGCCAA CCGCGATAGA
 851  TGATGTCGGG TGGGCTGTGC GACGAGATCA GCGCGGCCAG CAATACGTTG
 901  GTGTCGAGGA CGACCCGCAT TAATGCTCAC GCGCCCATTG CACCGCCTCA
 951  TCAATGAGGT CGTTCAGTTC TGTCTCACCC ATACCCACCG TAGCGGCTTT
1001  GGCTTGTTCC ACAGCCCGCT CGAAGAGGTA GGCGCGCACC GCATCCTCGA
1051  TGAAGCGTGA CAGGTCGCCC TTGCGACCGC CGCCTTGCGC GGCGATGAAC
1101  ATGCGGACGG ACTGATCCAC GTCCGGCGAT ACGGCGATGT TCCAGCGCAC
1151  GGTATTCATG AAGCTCTCTC CAATCAGAGT TTATGCACAT CGGTGTTTAT
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1201  ACACTCTTTA AGCGGTTTTT TTGCAACGGT TCAATAAAAC GATCGTTTTT
1251  ATGAACTCTG ATGGGGCGAT CGGCGCGAGG TGTCCGTAAG CCAACAAGCA
1301  GTCTGTAATA GTGGTTTGTA CAATCAAAGT TTGATATTAT TAAAAATTAA
1351  CGAGTAAAGC AAAATATTAC GATGAAAATC GGCTATGGCG CGTATCCACG
1401  CGCGAACAGA ACCCAGCCTT GCAGGTGGAC TCCCTCAAGG CGGCCGGCTG
1451  CGAGCGCATT TATCAGGACG TTGCGAGCGG CGCGAAGACC GCGCGCCCAG
1501  CGCTCGATGA ACTGCTGGGC CAG----------------------------
      ------------------------------------------------------
      ------------------------------------------------------
      ------------------------------------------------------
1701  ----------------------------------------------GGGGTTCGG
1751  GGAGCAATGG AACAGGGAAG TCAGTTAAGC CTTCACCAGT GCCGGTCTGT
1801  ACCTTCCATG CGGGCGTGGC TCTCTGAGAA ACAGTGGCGG TCATCCGCAC
1851  CGCAGGCGAT GTCGGTTAGC AAATCGTTCA CGGCTTCATC CAGCTCTTCA
1901  TCCGTGTCAT AGGGCACCTT CAGTTCATAT TCCCCGTTCG GCCGCCGCGT
1951  GGCATCGTAT TGGGCAAGAT CAAAAAACTC GACATGCTCG ATCGAGCGCT
2001  TCTTTCCGCG CACCGAACCT TGCTATTGTT CNCGATGC
```

Figure 4.6. The assembled nucleotide sequence obtained from pS32.1, pSS1, pSBXA3 and pSSPL3.
The position of the sequence is illustrated in Figure 4.4 as bases 5958-7995. The sequence in bold type shares similarity with *Xanthomonas* avirulence genes. The arrow indicates the start of the sequence sharing similarity with the *Xanthomonas campestris* pv. *citri* transposon. ISXC5. The dashes represent an unsequenced region of 518 bases.

4.3.2.3. The significance of the presence of the *avr* and transposon sequence

The aim of sequencing the extrachromosomal element was to locate typical phage genes that might aid in the characterisation of the novel *Xanthomonas* phage. With respect to this, the sequencing experiment was not successful, shedding no further light on the nature of the phage. One can only speculate on the origins of the fragments of *avr* and transposon sequence. These sequences may have been acquired from other extrachromosomal elements *e.g.* plasmids, or from the host chromosome, *e.g.* imprecise excision of prophage DNA.

It is not unknown for ssDNA bacteriophages to encode bacterial virulence factors, *e.g.* the cholera toxin encoded by bacteriophage CTXϕ (Waldor and Mekalanos, 1996). These

factors could then be transferred horizontally as well as vertically and confer an evolutionary advantage to their hosts. The novel phage, however, does not appear to possess an intact, contiguous *avr* gene sequence. With regard to the presence of the transposon sequence, it has been shown than transposons have played an important role in the evolution of temperate phages (reviewed by Reanney and Ackermann, 1982). Therefore, it is not unusual for phage genomes to harbour transposable elements, *e.g.* bacteriophage P1 (De Bruijn and Bukhari, 1978). The observation that it is only a partial transposon sequence - the 5' end being replaced by the *avr* sequence - may be a reflection of a scar(s) resulting from previous transposition events. The fact that transposons are capable of gene rearrangements and deletions, may also explain why the *avr* sequence is truncated, with the 3' and 5' sequences (situated at either end of the 3.5 kb of pSS1) appearing to converge.

Alternatively, the extrachromosomal element may be an indigenous plasmid harbouring the *avr* and transposon sequences. Given the fact that the double-stranded extrachromosomal DNA is 53.8 kb in size and cannot possibly be packaged into a capsid of 33 nm in diameter, even in single-stranded form, it seems plausible that the double-stranded form of the phage genome may have integrated into a plasmid. This integration event would explain why the ^{32}P-labelled single-stranded phage genome hybridised with an extrachromosomal element too large to be packaged into 33.5±4.5 nm particles.

CHAPTER 5

General Discussion and Conclusions

The causal agent of chocolate spot disease of crucifers, thought to be a strain variant of *X. c.* pv. *campestris*, the causal agent of black rot, was found to be associated with bacteriophage-like particles upon examination by EM. These particles, observed to occur extracellularly in the absence of plaque formation, were shown not to be vesicles originating from the bacterial membrane - a phenomenon not uncommon in Gram negative bacteria - as the protein profile and immunogenic properties of the particles differed from those of the bacterial host. This, together with the fact that plaque formation was extremely rare (≈5 occurrences of turbid plaque formation for the duration of project) and that particle production could not be eliminated from cultures of PCB 22 grown in the presence of the antibodies raised against purified particles, led to the conclusion that the particles associated with the chocolate spot pathogen were temperate phages engaged in a non-lytic life cycle. This conclusion led to the investigations described in Chapters 2 and 3, which unfortunately did not prove this assumption conclusively.

Although the gross morphology, *i.e.* the hexagonal shape and lack of tail, combined with the non-lytic mode of existence makes this phage similar to a tailless icosahedral phage (CP_2) isolated from *X. citri*, the latter phage has a considerably larger capsid diameter (70 nm) and readily forms turbid plaques (Wakimoto, 1967). Unfortunately the lack of literature detailing the characterisation of CP_2 prevented further comparisons.

The absence of a tail or modified vertex as well as the lack of obvious plaque formation raised the question of how the phage infects potential hosts and how it is released from the bacterium. Although numerous particles have been observed to be in contact with the cell wall, it can not be said with any certainty that the particles were in the process of infecting the bacteria via specific receptors. Attempts to infect the *X. c.* pathovars *graminis*, *vasculorum* and *campestris* ATCC 33437 and ATCC 33442 with either particles isolated in the usual manner (chapter 2) or phages isolated from turbid plaques were unsuccessful, possibly indicating the phage host range does not extend to these pathovars. This may have been due to the lack of appropriate receptors on

the cell surface. Alternatively there exists the distinct possibility that the particles are not infectious: the phage genome could be a fossil remnant of a long-established lysogeny, which retains the ability to produce particles but not infectious genomes. Growth of the host PCB 22 in the presence of anti-phage serum has already shown that the production and release of the phage is not dependent upon the presence of extracellular particles: this would appear to indicate that all particles produced originate from integrated genomes, and that the phage is defective. Other possible explanations for the lack of plaque formation are that: (i) the phage may behave like a symbiont, continuously extruded through the cell wall, in a manner similar to that of filamentous phages, or (ii) the rate of bacterial reproduction is either in equilibrium with cell lysis or exceeds it, thereby masking the usually visible effects of phage infection and lysis on solid media.

Early data of potential evolutionary significance came from the serological comparisons of phage coat proteins and proved most useful in grouping ϕX174-like phages (Reanney and Ackermann, 1982; Denhart, 1975). Serological data obtained using the anti-phage serum indicate that the novel phage shares similarity with the *X. c.* pv. *campestris* phage, HT_{3h}. The novel phage antiserum recognised a 21.8 kDa protein in both phages which is localised in the capsid as determined by ISEM. It is rather surprising to find that this novel phage has similarity to a phage so dissimilar in morphology and nucleic acid content. Based on these results one could begin to speculate on the possible evolutionary origin of the phage infecting the chocolate spot pathogen. The loss of the tail structure might have occurred as a result of the survival of a mutant form of the tailed phage; similarly the change from a lytic to a temperate nature which would allow a phage defective in the infection process to be perpetuated from one generation to the next. The single-stranded genome of the novel phage is not so easily explained. It may have been a survival strategy since an advantage of being single stranded is that twice as many progeny can be produced from the same replication enzyme pools. One has to take into account, however, that similarities do not necessarily mean that a close evolutionary relationship exists. Thus, the partial serological similarity between HT_{3h} and the novel phage may reflect a shared evolutionary origin, or possibly the sharing of a genetic element (module) derived by a recombination event in accordance to Botstein's (1980) modular theory of phage evolution.

Since the nucleic acid of the purified particles was determined to be ssDNA, it was assumed that a double-stranded replicative intermediate would be present in the bacterial host. An extrachromosomal dsDNA element was in fact isolated, and since it hybridised strongly to the single-stranded phage genome, it was tentatively designated the RF of the mature phage genome. However, its high molecular weight (53.8 kbp) cast doubts upon this, even though having a single-stranded genome means that at least twice as much genetic information as ssDNA than dsDNA can be packaged and that this amount can be further increased due to the formation of hydrogen bonds between complementary nucleotides and ultimately, more compact molecules (Watson *et al.* 1987). Since capsid size places constraints upon the amount of DNA to be packaged, and given the diameter of the phage is 33.5±4.5 nm, it is not possible that 53.8 kb of ssDNA could be packaged within it. Thus, we decided to sequence the putative RF, in order to identify any phage-specific sequences. No phage genes were identified in those clones sequenced, but fragments of a *Xanthomonas avr* gene interrupted by part of a transposon sequence were found. The truncated transposon sequence might possibly be a reflection of previous transposition events at that particular site.

It has been shown that the host range of the chocolate spot pathogen closely resembles that of *X. c.* pv. *campestris* and that it too elicits the hypersensitive response on tomato (Machaba, 1998). The hypersensitive response is the result of the interaction of a bacterial *avr* gene with a corresponding resistance gene in the plant (Keen, 1990). Although the *avr* sequence identified does not appear to be a contiguous, full length gene, it is still possible that it may retain its function. The deletion of a certain number of repeats in the *avr*Bs3 gene is known to effect changes in the specificity of the *avr* gene (Herbers *et al.* 1992). Yang and Gabriel (1995) demonstrated that intragenic recombination amongst the 102 bp homologous repeats in the *X. citri pth*A gene is possible and may yield smaller functional genes which occasionally results in the gain of new pathogenic phenotypes and *avr* specificities. Hybridisation of the extrachromosomal element isolated from PCB 22 to the pSS1 insert containing part of the *avr* sequence (see Figure 3.7b) showed that it hybridised to 2 bands of 12.7 and 9.9 kb in size. Cloning and sequencing of these 2 bands (the homologous region of which may be isolated by further restriction endonuclease digestion) would reveal the extent of the *avr* sequence present in the chocolate spot pathogen. If this gene proves to be a truncated version of the *avr*Bs3-like genes or harbours significant mutations/deletions in the central repeat region, this would

certainly contribute to the explanation as to why the chocolate spot pathogen would so closely resemble *X. c.* pv. *campestris* with respect to host range, phenotypic characteristics and 16S rDNA sequences, yet elicits notably different symptoms on crucifers.

Although phages have been known to harbour transposons (De Bruijn and Bukhari, 1978) as well as to play a role in the transfer of virulence determinants by encoding a variety of toxins (Barksdale and Arden, 1974; Hayashi *et al.* 1993; Waldor and Mekalanos, 1996), it is my belief that these sequences do not form part of the phage genome. It is most probable that the *avr* and transposon-like sequences are part of an indigenous plasmid, and that the reason the ssDNA genome of the phage hybridised to the extrachromosomal element, is because the authentic RF had integrated into the plasmid. It is not unknown for phages to integrate into plasmids: the *Bacillus thuringiensis* phage, J7W-1, is stably integrated into the indigenous plasmid, pAF101 (Kanda *et al.* 1989). Integration into a plasmid means that the size of the ssDNA packaged into the phage head is smaller than the 53.8 kb of the entire extrachromosomal element and therefore more likely to fit into a capsid of 33.5±4.5 nm in diameter. In theory, determining whether the phage genome forms only a part of the extrachromosomal DNA would prove to be relatively easy. The extrachromosomal DNA would need to be digested by a restriction endonuclease and then probed with the ssDNA isolated from the purified particles. The reason this has not yet been done is because of the great difficulty in isolating the ssDNA.

The following points were demonstrated by Dai *et al.* (1987) for the ssDNA *Xanthomonas campestris* pv. *citri* phage, Cflt:

(i) the integrated and free form of the phage genome can coexist in the host for long periods,

(ii) cells containing free RF yielded a higher phage titre,

(iii) the phage yield decreased after serial transfer of large yellow colonies, as a result of these cell containing only the integrated prophage.

These aspects were not actively investigated during the characterisation of the novel phage. However, only one species of extrachromosomal DNA was consistently isolated, indicating that no "free" form of the phage genome existed within the host. In addition it was noted that phage and extrachromosomal DNA yields were highest during the first three transfers of colonies to maintain cultures and that yields occasionally decreased upon subsequent transfer. The decrease

may have been due to the transfer of yellow mucoid colonies. Although no free phage DNA was found in the cells, one could still hypothesize that a process similar to that described by Dai *et al.* (1987) might be occurring in PCB 22. The difference between the novel phage and the ssDNA phages described by Dai *et al.* (1987) is that the latter phages integrate into the host chromosome, whereas it appears that the novel phage may have integrated into a plasmid. Alternatively, since an important part of phage evolution is thought to have taken place by rearranging and recombining modular elements (blocks of linked genes or individual genes) (Botstein, 1980; reviewed by Reanney and Ackermann, 1982), the particles and the DNA that were isolated and characterised could be – as noted earlier – non-infectious and defective "fossil remnants" resulting from an unsuccessfully deleted and/or recombined remnant of a lysogenic viral genome. This situation has parallels with the production of retrovirus-like non-infectious particles by certain mammalian cell lines (Coffin, 1990).

In summary, I have accomplished the following during the course of this project:

(i) morphologically, physically and antigenically characterised a novel class of particles containing ssDNA, which appear to be a bacterial virus associated with a strain variant of *X. campestris* pv. *campestris*;

(ii) showed that the viral ssDNA shares some sequences with a 54 kb plasmid-like dsDNA element isolated from the bacteria;

(iii) demonstrated that this plasmid contains an *avr*-like sequence which appears to be interrupted by a transposon.

The interesting combination of characteristics provided by the morphology, nucleic acid content and mode of existence of the novel phage indicates that further research may possibly elucidate a new feature of phages by conclusively proving that this phage is indeed capable of a form of neolysogenization. It would also be interesting and no doubt invaluable to know whether the presence of the phage in the bacteria has any effect on the severity of the disease caused by the pathogen, and whether in fact the phage is defective.

APPENDIX

APPENDIX A

Standard buffers and solutions

A.1. Standard Mineral Base Medium

$Na_2HPO_4.2H_2O$	: 4.75 g
KH_2PO_4	: 4.53 g
NH_4Cl	: 1.0 g
$MgSO_4.7H_2O$	: 0.5 g
5% (w/v) ferric ammonium citrate	: 1 ml
0.5% (w/v) $CaCl_2$	: 1 ml
Add distilled water up to	: 900 ml

Autoclave, then add glucose to a final concentration of 0.3% (w/v)

A.2. Phosphate Buffered Saline

Na_2HPO_4	: 4.26g
KH_2PO_4	: 2.27g
NaCl	: 8.0g
$MgSO_4$	: 5 ml (from 5 M stock solution)

distilled water up to : 1 liter

Adjust the pH to 7.2 with NaOH

A.3. TEN Buffer

1 M Tris-Cl pH 8.0	: 10 ml
0.5 M EDTA	: 2 ml
NaCl	:
25% SDS	: 20 ml
distilled water	: 968 ml

A.4. Phosphate buffered tryptone (PBT)

Tryptone	: 10 g
Yeast extract	: 5 g
NaCl	: 5 g
Glucose	: 1 g

Distilled water : to 1 L

Soft agar and agar plates were prepared with broth containing 0.6 and 1.6% agar, respectively.

APPENDIX B

Standard Methods

B.1. Rate zonal density gradient centrifugation

Preformed sucrose gradients were made by adding 18ml each of 5% and 20% sucrose solutions to separate chambers of a gradient maker. The solutions were mixed by mechanical stirring and the gradient made in ultraclear tubes using a peristaltic pump. Four hundred microlitres of the phage preparation was layered on top of the preformed gradient. The gradients were loaded on a Beckman SW28 swinging bucket rotor and centrifuged at 25 000 rpm and a temperature of 4°C for 2.5 hours. Gradients were observed in the dark with a fibre-optic light source shining into the gradient from above and the phage band removed using a pasteur pipette with a tip bent at a right angle. The sucrose was removed from the samples by diluting the solutions 1:4 with PBS pH 7.2. and concentrating the phage by ultracentrifugation at 40 000 rpm in a 40 Ti rotor for 90 min at 4°C.

Rate zonal gradient reagents

5% sucrose 5% (w/v) made in PBS pH 7.2

20% sucrose 20% (w/v) made in PBS pH 7.2

B.2. Isopycnic density gradient centrifugation

One hundred microlitres of purified phage was layered onto 5ml of caesium chloride with an initial density of 1.336g/cm^3 in ultraclear tubes. The tubes were placed in rotor buckets and loaded on an SW50.1 swinging bucket rotor. The gradients were centrifuged at 44 000 rpm for 16 hours at 20°C in a Beckman ultracentrifuge. The gradients were observed in the dark, using a fibre-optic light source. The gradients were fractionated by puncturing the bottom of the tubes. The absorbance of each fraction was measured at 260nm in a Beckman DU-64 spectrophotometer. The refractive index was measured using an Atago hand-held refractometer, and the buoyant density determined from Beckman standard tables (Griffith, 1979).

B.3. Protein Methods

B.3.1. The Bradford protein assay for protein quantitation

The following amounts of 1 mg/ml BSA and 0.15 M NaCl were aliquoted (in duplicate) into eppendorf tubes.

BSA	NaCl
2.5 µl (2.5 µg/ml)	97.5 µl
5 µl (5 µ/ml)	95 µl
10 µl (10 ug/ml)	90 µl
15 ul (15 ug/ml)	85 µl
20 ul (20 ug/ml)	80 µl

100 µl of the protein sample of unknown concentration was added (in duplicate) to an eppendorf tube. 1ml of Coomassie Brilliant Blue (Appendix B.3.3) was added to the standard and sample tubes. The samples were vortexed for 5 seconds and then allowed to stand at room temperature for 5 min. The absorbance of the samples was determined at 595 nm. Using the values obtained for the protein standards, a standard curve of A595 versus protein concentration was plotted. The concentration of the phage samples were determined from the graph, using the equation for a linear graph: y= mx + c, where y= absorbance at 595nm, m= gradient, x= the amount of protein, and c= the constant

B.3.2. SDS-PAGE

Protein samples were denatured in 62.5 mM Tris-Cl (pH 6.8), 5% SDS, 5% β-mercaptoethanol by mixing the phage sample with an equal volume of disruption buffer and heating for 10 min at 100°C. Pharmacia low molecular weight markers were used as molecular weight standards. The disrupted proteins were elactrophoresed through polyacrylamide gels using the discontinuous buffer system of Laemmli (1970). Large polyacrylamide gels were run in a vertical slab gel apparatus (Hoefer SE 600, Hoefer Scientific Instruments, San Fransisco) and small polyacrylamide gels were run in a vertical slab gel apparatus ("mighty small", Hoefer Scientific Instruments, San Francisco). Large gels were run overnight at 20mA (constant current) and a temperature of 4°C with constant stirring. Small gels were run at 25mA (constant current) for approximately 2 hours at room temperature.

SDS-PAGE reagents

Resolving gel buffer: 1M Tris-Cl, pH 8.8

Stacking gel buffer: 1M Tris-Cl, pH 6.8

Acrylamide solution: 40% Acrylogel BDH (39:1 acrylamide:bis)

Electrophoresis buffer: 0.12M Tris, 1.25M glycine, 0.5% SDS

Disruption buffer: 125mM Tris-Cl pH 6.8, 10% (v/v) β-mercaptoethanol, 10% SDS, 10% (v/v) glycerol, 0.01% bromophenol blue.

Ammonium persulphate: 10% (w/v)

Protein gel preparation

Stock solution	Resolving gel (12%)		Stacking gel (5%)	
	Large	Small	Large	Small
Acrylogel	6.0 ml	3.0 ml	1.26 ml	0.63 ml
Water	6.06 ml	3.03 ml	7.28 ml	3.64 ml
Resolving gel buffer	7.5 ml	3.8 ml		
Stacking gel buffer			1.26 ml	0.63 ml
10% (w/v) SDS	200 µl	100 µl	100 µl	50 µl
50% ammonium persulphate	60 µl	30 µl	30 µl	15 µl
TEMED	20 µl	10 µl	10 µl	8 µl

B.3.3. Coomassie Brilliant Blue protein gel stain

The gels were stained in stain solution for 2-16 hours with gentle agitation at room temperature.The gels were destained in several changes of destain solution for 8-16 hours.

Stain reagents

Stain: 0.2% (w/v) Coomassie Brilliant Blue, 45% (v/v)distilled water, 45% (v/v) methanol, 10% (v.v) glacial acetic acid

Destain: 25% (v/v) methanol, 10% (v/v) glacial acetic acid, 65% distilled water

B.4. Immunological tests

B.4.1. Immunoelectroblotting (IEB)

Immunoelectroblotting was performed essentially according to the method of Towbin *et al.* (1979) using a semi-dry blotting apparatus (Omeg Scientific, SA). After electrophoresis, nitrocellulose membrane (pore 0.45 μm, MSI) presoaked in transfer buffer and the polyacrylamide gels were placed between presoaked filter paper. This "sandwich" was placed between nappy liners (Johnson & Johnson) soaked in transfer buffer, and placed in a semi-dry blotting apparatus. Transfer was carried out at 500mM for 40 min constant current. After transfer of the proteins to the nitrocellulose membrane, the membranes were placed in blocking buffer to allow non-specific protein binding sites to be blocked. Anti-phage serum were appropriately diluted (1:200) in blocking buffer and the membranes were allowed to incubate for one hour at room temperature. The blots were washed three times for 5 min in wash buffer. The blots were then incubated in a dilution of goat anti-rabbit gamma globulin linked to alkaline phosphatase (GAR-AP) (1:1000) in blocking buffer and incubated for one hour at room temperature. The blots were washed as before and incubated in fresh substrate buffer containing 0.33 mg/ml nitro blue tetrazolium (Sigma), 0.17 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Sigma), 0.33 % (v/v) N, N-dimethylformamide. The membranes were left in the substrate for 5-10 min and then washed in water and left to dry before being photographed.

Immunoassay reagents

Transfer buffer : 25mM Tris base; 192mM glycine; 20% (v/v) methanol

Blocking buffer : 5% (w/v) fat free milk powder in PBS

Wash buffer : 0.05% Tween 20 in PBS

Substrate buffer : 100mM Tris; 100mM NaCl; 5mM $MgCl_2$

Nitro blue tetrazolium : 75mg/ml (w/v) in 70% dimethyl formamide

5-bromo-4-chloro-3-indolyl-phosphate: 50 mg/ml (w/v) in 100% dimethyl formamide

B.4.2. Indirect Enzyme-linked immunosorbent assay (ELISA)

200 μl of the phage samples were loaded into the wells of the trays and incubated at 37°C for 1 hour. The samples were removed and the wells were washed thrice with PBS-Tween for 5 min. The trays were flooded with PBS-Tween-milk powder solution and left at room temperature for 10 min. The trays were then dried and 200 μl of a 1: 500 dilution of the anti-

phage serum was added to each well and incubated at room temperature for 1 hour. The wells were washed as before. 200 µl of a 1:1000 dilution of goat anti-rabbit gamma globulin linked to alkaline phosphatase was added to the wells. The trays were incubated overnight at 4°C in a humid box. The wells were washed and 300 µl of substrate solution added. The trays were left at room temperature for the enzyme-catalysed reaction to occur: the colourless substrate becomes a bright yellow colour if antigen is present. The reaction was stopped by the addition of 50 µl/well of 3 M NaOH. The results were read on a Titertek Multiskan multichannel colorimeter at 405 nm.

ELISA reagents:
PBS-Tween : 4.26g Na_2HPO_4, 2.27g KH_2PO_4, 8.0g NaCl, 0.3% (v/v) Tween 20
PBS-Tween-milk powder : as above except for the addition of 5% (w/v) milk powder
Substrate : 1 mg/ml p-nitrophenyl phosphate in 10% diethanolamine pH 9.8

B.5. Electron Microscopy

B.5.1. Negative staining

Samples were stained according to the method of Griffin (1990). Carbon-coated copper grids were floated on the surface of 30µl of each sample for 20 minutes. Excess sample was removed by gently washing the grid with water. The grids were dried and then stained with 2% (w/v) uranyl acetate (pH 7.4) for 2 minutes. Excess stain was removed before viewing on the electron microscope

B.5.2. Thin-sectioning of bacteria

The bacteria were fixed in 2% glutaraldehyde for 24 hours. Thereafter it was washed twice with PBS and post-fixed with 1% osmium tetroxide for 1 hour. The samples were washed with buffer, followed with distilled water. The fixed samples were dehydrated in an ethanol series: 30%, 50%, 70%, 80%, 90%, 95% and 2x 100%, followed by 2x 100% acetone. The samples were finally put into a 1:1 mixture of acetone:Spurrs resin and left to mix for 16 hours. After 16 hours the resin:acetone mixture was changed to 2:1 and left to mix for 6 hours. This mixture was then replaced with 100% Spurrs resin, allowed to mix for/infiltrate for 16 hours, after which the existing mixture was replaced with 100% Spurrs resin. After 5 hours the sample was put into capsules and this was topped up with fresh 100% Spurrs resin and allowed to cure for 16 hours at 60°C. Thin sections (90 nm) were cut on a Reichert

Ultramicrotome and were picked up on 200 mesh nickle grids. The sections were viewed in a ZEISS EM109 electron microscope.

B.5.3. Immunosorbent electron microscopy

The grids were floated on 0.02 M glycine in PBS for 3 min, 1% BSA in PBS for 5 min and phage antiserum diluted with PBS-BSA for 2 hours. The grids were washed 5 times for 1 min each cycle. The grids were stained with 2% uranyl acetate. Alternatively, the grids containing the thin sections were floated on colloidal gold diluted with PBS-BSA for 2 hours. Thereafter the grids were rinsed twice with PBS and 5 time with water. Finally, the grids were stained with uranyl acetate and lead citrate.

B.6. Preparation of bacterial genomic DNA

A 100 ml culture was grown to saturation at 30°C with shaking. Bacterial cells were collected by centrifuging samples for 5 min at 5000 rpm. Cells were resuspended in 9.5 ml TE (10 mM Tris, 1 mM EDTA pH 8.0) buffer. Cell lysis was performed by adding 500 µl of 10% SDS and 50 µl of 20 mg/ml proteinase K and incubating the sample for 1 hour at 37°C. 1.8 ml NaCl was added and the sample was mixed thoroughly before the addition of 1.5 ml CTAB/NaCl solution. After an incubation of 20 min at 65°C, and equal volume of chloroform/isoamyl alcohol was added, and the sample was centrifuged at 7000 rpm for 5 min to separate the phases. The aqueous supernatant was transferred to a fresh tube using a wide-bored pipette, and the DNA was precipitated out of solution with 0.6 volumes of isopropanol. The DNA was pelleted at 10 000 rpm for 10 min, and washed with 70% ethanol. The pellet was resuspended in 500 µl TE, pH8 and the concentration of DNA was determined spectrophotometrically by measuring the A260 between 210 nm and 310 nm of a 1 in a 100 dilution. The relationship of A260 = 1 for 50 µg/ml DNA was used.

B.7. Large scale isolation of extrachromosomal DNA

A 400 ml culture was grown overnight with shaking at 37°C. The cells were harvested in a GSA centrifuge tube at 6000 rpm for 10 min at 4°C. The pellet was resuspended in 4 ml of Solution I, transferred to an SS34 centrifuge tube and left for 10 min at room temperature. 8 ml of Solution II was added. The tube was rolled gently to mix the solutions and was incubated on ice for 10 min. 6 ml of Solution III was added, the tube was mixed well and incubated for 10 min on ice. The contents of the tube was centrifuged at 10 000 rpm for 10 min and the supernatant transferred to a new SS34 tube. An equal volume of isopropanol

was added and the tube was left at room temperature for 10 min before centrifugation at 15 000 rpm for 15 min. The pellet was resuspended in 4.2 ml TE buffer pH 8.0. 5.2 g of CsCl and 200 • l of 10 mg/ml of ethidium bromide was added. The tube was centrifuged at 10 000 rpm for 10 min, and the refractive index adjusted to 1.396. The sample was sealed in a Beckman Quickseal ultracentrifuge tube (5 ml) and centrifuged overnight at 55 000 rpm in a Beckman Vti 65 rotor at 15° C. The extrachromosomal DNA was extracted in the smallest possible volume from the side of the tube using a 1 ml syringe and a 1.2 mm gauge needle under long wavelength UV light (350 nm). The ethidium bromide was extracted at least 3 times using equal volumes of salt saturated isopropanol. Two volumes of water were added to the DNA solution. One volume of isopropanol was added to this, and the DNA was pelleted at 13 000 rpm for 10 min. The DNA was resuspended in 100 • l TE pH 8.0 and the concentration of DNA was determined spectrophotometrically as described in A1.

DNA isolation reagents

TE: 1 mM EDTA, 10 mM Tris pH 8.0

Sodium Chloride (NaCl): 5 M NaCl

CTAB/NaCl: 4.1 g NaCl, 10 g CTAB (hexadecyltrimethyl ammonium bromide) in 100 ml distilled water

Solution I: 50 mM glucose, 25 mM Tris-HCL, pH 8.0

Solution II: 0.2 M NaOH, 1% (w/v) SDS

Solution III: 5 M potassium acetate, pH 4.8

B.8. Fluorescent staining of bacteriophage nucleic acid

Characterisation of the phage nucleic acid was performed by slightly modifying Bradleys' (1966) method. Small droplets (10 ml) of the purified phage were placed on a microscope slide and dried in a stream of warm air. The dried "spots" were fixed by placing the slides in a petri dish containing Carnoys' fluid for 5 min at room temperature. They were then removed, washed briefly in absolute ethanol and dried in a stream of warm air. The fixed slides were placed in 0.01% (w/v) acridine orange in modified McIlvaines' buffer at pH 3.8 for 5 min. They were then briefly rinsed twice in McIlvaines' buffer, after which the slides were soaked in 0.15 M disodium hydrogen phosphate solution for 15 min. Excess liquid was shaken off and the colours of the spots observed under UV light (254 nm). This treatment indicated the type and strandedness of the nucleic acid. Confirmation of the results was

obtained by placing the slides in a dish of tartaric acid under the UV lamp. Colour changes were observed over 2-5 min without removing the slide from the solution.

Reagents for nucleic acid analysis

Disodium hydrogen phosphate (Na_2HPO_4): 0.15 M

Tartaric acid: 0.1 M

Modified McIlvaines' citric acid and phosphate buffer, pH 3.8: 0.1-M citric acid, 6 ml; 0.15 M-Na_2HPO_4

Acridine orange: 0.01% (w/v) in buffer: 1% (w/v) acridine orange solution, 0.1 ml; modified McIlvaines' buffer, 10 ml

B.9. Restriction endonuclease digestion of DNA

Restriction digests were performed as outlined by Sambrook *et al.* (1989). Restriction digests typically contained 2 U of enzyme per 500 ng of DNA. All digests (except for the bacterial genomic DNA, which were incubated overnight) were left at 37°C for 2 hours unless otherwise stated by the manufacturer.

B.10. DNA gel Electrophoresis

Agarose gel electrophoresis was performed in submerged horizontal slab gels. Gels were electrophoresed at 40 V overnight or 100 V for 4-6 hours. Hoefer "mini-gel" apparatus (model HE 33, Hoefer Scientific Instruments, San Francisco) were used for quick runs and electrophoresed at 100 V for 1-2 hours. DNA was sized against λ DNA digested with *Pst*I or *Eco*RI. Tris-borate-EDTA was used for analytical gels, while Tris-acetate-EDTA was used for gels from which DNA bands were to be excised for purification by Geneclean (Bio 101). Agarose (0.3%-1%) was dissolved in TBE or TAE by heating. The gels were cooled to approximately 50°C before pouring and run in the appropriate buffer. Ethidium bromide (0.5 μg/ml) was added to the gel for visualisation of the nucleic acid on a 254nm transilluminator. When DNA was to be recovered, the gels were visualised on a 365nm transilluminator.

DNA gel electrophoresis reagents

Tris-EDTA:	10 mM Tris-Cl, 1 mM EDTA, pH 7.6
Tris-borate-EDTA:	89 mM Tris, 89 mM boric acid, 2 mM EDTA
Tris-acetate-EDTA:	40 mM Tris-acetate, 2 mM EDTA pH 8.0

Loading buffer: 10% (w/v) SDS, 0.02% bromophenol blue in TE buffer

Ethidium bromide: 1% (w/v)

B.11. Geneclean

DNA fragments were removed from TAE gels and purified using the Geneclean II Kit (Bio 101 Inc.) as per the manufacturers instructions. Briefly, the procedure is as follows: the relevant DNA bands were cut out from TAE gels and placed in 1.5ml eppendorfs. The fragment was weighed, three times the volume of 6 M sodium iodide was added and the gel was melted at 50°C for 5min. 5 µl glassmilk was added and the solution shaken vigorously and placed on ice for 5min. The eppendorf was centrifuged at 10 000 rpm for 30 sec. The supernatant was removed and the pellet washed three times with NEW wash solution. The NEW was solution was removed and the glassmilk pellet was resuspended in 10ul TE buffer and placed at 50°C for 5min. The eppendorf tube was centrifuged at 10 000 rpm for 30 sec and the TE buffer removed and placed in a new eppendorf tube.

B.12. Removal of the 5′ phosphate group of the vector

Following the removal of the restriction enzymes from a digested sample of pSK by phenol/chloroform extraction, the DNA was salt precipitated in ethanol. The DNA was resuspended in 50 µl containing 5µ l CIP and 5 µl (1x) CIP dephosporylation buffer. The reaction mix was incubated at 37°C for 30 min. The reaction was stopped by heating at 75°C for 10 min. The DNA was ethanol precipitated and resuspended in TE buffer pH 8.0.

B.13. Transformations

E. coli was made competent by the rubidium chloride protocol of Chung and Miller. 5 µl of the ligation mix was transformed into 100 µl competent JM109 *E. coli* cells by placing the cells on ice for 10min and then heat shocking them at 37°C. The cells were plated on LA-IPTG-XGal plates for overnight incubation at 37°C.

Transformation media

LA-IPTG-XGal plates: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar made to 1 L and autoclaved. Cool to 50°C, add ampicillin to 100 µg/ml, 20 mg/ml of X-gal, 200 mg/ml of IPTG and pour plates.

B.14. Nucleic acid hybridisation

B.14.1. Alkali DNA transfer

The DNA fragments to be transferred were separated by 0.6% agarose gel electrophoresis in TAE buffer. The DNA was depurinated by soaking the gel in 0.25 M hydrochloric acid (HCl) for 15 min with gentle shaking. The gel was briefly rinsed in distilled water. A capillary transfer system was set up. A Whatman 3MM filter wick was wetted with 0.4 M NaOH and placed over a glass bridge so that the wick ends were touching the bottom of a reservoir tank containing 0.4 M NaOH. The gel was placed on the wick, on top of which was placed a wetted nylon Hybond N+ membrane and 3 sheets of Whatman 3MM filter paper. A stack of absorbent tissue 10 cm in height was placed on top of the membrane, along with a 1 kg weight to compress the tissue. The DNA was fixed to the membrane by UV crosslinking on a 254 nm transilluminator for 5 min.

B.14.2. Nucleic acid hybridisation of DNA to a DIG-labelled probe

Prehybridisation, hybridisation and detection procedures were performed according to the instructions of the manufacturer of the Nonradioactive DNA Labelling and detection Kit (Boehringer Mannheim). The membrane was allowed to prehybridise for a minimum of 4 hours in prehybridisation buffer in a plastic container at 65°C with gentle shaking. The DIG-labelled DNA probe (25 ng DNA/ml buffer) was heat denatured at 100 °C for 10 min, added to the membrane and incubated overnight at 65°C. The membrane was washed twice for 5 min each in 2X SSC/0.1% SDS at room temperature, and then washed twice for 15 min at 65°C in 0.1X SSC/0.1% SDS. Subsequent steps were performed at room temperature. After post hybridisation washes, the membrane was equilibrated in buffer 1 for 1 min and then blocked in buffer 2 for 40 min. The membrane was then incubated in anti-DIG-alkaline phosphatase diluted 1 in 10 000 in buffer 2 for 30 min. The membrane was removed from the antibody solution and washed twice for 15 min each in buffer 1. The membrane was placed in buffer 3 for 2 min, and then in a 1:200 dilution of CSPD in buffer 3 in the dark for 5 min. The membrane was blotted dry on paper towel and placed in a plastic bag and incubated at 37°C for 15 min. The membrane was then exposed to autoradiographic X-ray film.

B.14.3. Hybridisation of DNA to a ^{32}P-labelled probe

The membrane was prehybridised for 30 min in Church prehybridisation buffer (CHB) at 65°C with agitation. The probe DNA (15 ng/ ml CHB, where the specific activity of the probe was 2.88 x 10^8 cpm/µg DNA) was heat denatured at 100°C for 10 min and added to the membrane along with fresh CHB (50 µl/cm^2 membrane). The membrane was incubated overnight at 65°C with agitation. The membrane was washed twice in wash buffer A at 65°C for 10 min, followed by 4 washes in wash buffer B. The SDS was removed by a brief rinse in distilled water. The membrane was sealed in a plastic bag and exposed to X-ray film for 3 days at -70°C.

Reagents for radioactive Southern hybridisation

1 M Na_2HPO_4, pH 7.2 (PB stock solution, 1 L): 134 g $Na_2HPO_4.7H_2O$ (0.5 M), 4 ml of 85% H_3PO_4

Church prehybridisation buffer (CHB): 0.5% milk powder, 0.5 M PB, 1 mM EDTA, 7% SDS

Church hybridisation buffer: as for the pre-hybridisation buffer, except the milk powder was omited

Wash buffer A: 5% SDS, 40 mM PB, 1 mM EDTA

Wash buffer B: 1% SDS, 40 mM PB, 1 mM EDTA

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