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THE *VIBRIO* SP.2 PHAGE $\alpha 3$: A PHYSICAL CHARACTERISATION

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A Dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science.

Johannesburg, 1981

TO WHOM IT MAY CONCERN

I declare that the experimental work contained herein is my own unaided effort. Members of the MRC Microbial Genetics Unit gave advice on the mastering of electron microscopy techniques and some results were obtained at this Unit by the author.

No part of this dissertation has been submitted in the past nor is to be submitted in the future for a degree in any university.

Pauline T Jones

Johannesburg

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SUMMARY

In this study on the physical characteristics of the Vibrio phage α3 it was found that the buoyant density (in CsCl) was 1,506g.cm.$^{-3}$. The α3 genome length was determined as 51,98 ± 1,29 kilobases and its molecular weight as 34,66 x 10^6 daltons. Partial denaturation studies showed the α3 genome to be non-randomly circularly permuted over 27 percent of its genome length. Duplex studies revealed a terminal repeat of 2.4 ± 0,8 percent of the genome length, which, together with the maximum permutation of 27 percent, gives a probable concatemer length of 11 ± 3 genomes. Two populations of duplex molecules were observed viz. homo- and heteroduplexes. The phage α3 lysate used in the initial studies reported here therefore appears to consist of two phage populations, one having a relative insertion (or deletion) of 2,0 ± 0,4 percent of the genome length with respect to the other. Comparative restriction analysis (using Eco R1, Bam H1 and Hind III) between the DNA of the α3 stock and that of single plaque isolates was performed.

Hind III restriction endonuclease analysis revealed a band (Hind α , 7,6Md) which was present in 4 of the single plaque isolates but not in 2 others. This band was also present in the restriction pattern of the stock α3 lysate.

The Hind III restriction pattern of the α3 stock DNA also displayed a faint band (Hind C, 6,4Md), generally considered to represent a permuted fragment. Thus restriction analysis results are in agreement with a circularly permuted genome for phage α3 and proved successful separation of the individual members of the original dual population.

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ABBREVIATIONS

BAC	Benzyldimethylammonium chloride
BSA	Bovine serum albumin
EDTA	Ethylenediaminetetra-acetic acid sodium salt
g.	Gravity
h.f.t.	High frequency transducing
kb.	kilobases
log.	logarithmic
LSA	luria salt agar
LSB	Luria salt broth
LSSA	Luria soft salt agar
Md.	Megadalton
µg	microgram
µl	microlitre
µm	micrometre
ml	millilitre
m.o.i.	multiplicity of infection
O.D.550	Optical density at 550 n.m.
PEG	Polyethylene glycol 6000
p.f.u.	Plaque forming units
r.p.m.	Revolutions per minute

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I : INTRODUCTION

Vibrio sp.2 (recently reclassified; Robb *et al.*, 1980) was isolated from raw hides (Thomson *et al.*, 1972). It is a gram-negative, collagenolytic, halotolerant bacterium, which was found to contain a cryptic prophage (Thomson and Woods, 1974). Cryptogenicity appears to follow the model proposed for *Proteus mirabilis* (Krizsanovich, 1973). This model suggests that prophage integration causes disruption of the repressor gene (leading to loss of superinfection immunity) and that the phage excision apparatus (or some late phage functions) is defective. No extensive deletion is postulated in the cryptic prophage of *Vibrio* sp.2 (Thomson and Woods, 1974).

Phage α3 is inducible by various mutagens e.g. mitomycin C and N-methyl-N-nitroso-N^1-nitrosoguanidine (Thomson and Woods, 1974). Phages α3a and α3b are considered very closely related to each other and are postulated as variants of phage α3 (Thomson and Woods, 1974). Two other α phages (α1 and α2), infecting and lysing *Vibrio* sp.2, were isolated. Phage α1 was obtained from a raw hide soak and phage α2 from ultraviolet induction of *Vibrio* sp.9 (Thomson *et al.*, 1972).

The transducing ability of the α phages was tested with a view to developing a genetic mapping system in *Vibrio* sp.2 and ultimately to probe the genetics of collagenase production (Woods and Thomson, 1975). It was found that the four phages

studied were capable of generalised transduction of six markers (arg, cys B, met, pro and trp), albeit at very low frequencies. The observed generalised transducing system was postulated to be unique, in that it gave rise to unstable transductants.

A new phage-host system, in which phage ∝3a grows on stationary phase Vibrio sp.2 mutant strains, was found (Woods, 1976). The bacterial mutation allowing such growth was found to be an altered RNA polymerase, which can also confer cellular resistance to rifampicin (Robb et al., 1977). It has since been noted that these bacterial mutations preclude phage growth on logarithmic (log.) phase cells (Thomson, personal communication). It was postulated (Robb et al., 1977) that the altered RNA polymerase may undergo a change in template specificity allowing phage growth in stationary phase cells (but not in log. phase ; Thomson, personal communication). In addition, it is thought that the phage itself could play a role in the recognition of logarithmic and stationary phase polymerases (Robb et al., 1977).

At present, the nature of the ∝ phage genome is known to be double stranded DNA (Thomson and Woods, 1974), but more detailed analysis has not been carried out. Due to the unsolved problems involving failure to isolate high frequency transducing phages (h.f.t.), growth on stationary phase Vibrio sp.2 and the use of the ∝ phages as cloning vectors, it appeared imperative that the physical characteristics of the phage and its genome be further investigated.

II: PHYSICAL STUDIES OF VIRUSES AND THEIR RELEVANCE TO VIRAL GENETICS

The main physical parameters of the α phage discussed in this dissertation are : phage buoyant density, DNA microscopy (covering molecular weight determination, partial denaturation mapping and duplex formation) and restriction analyses. These topics are considered and related to generalised transduction, an important feature of phage α3.

BUOYANT DENSITY OF MATURE PHAGE PARTICLES. The phage buoyant density is affected by the nature of its capsid, its structure and the type of nucleic acid. Weigle and Meselson (1959) were able to deduce the protein composition of the phage λ capsid from information on its buoyant density and the base composition of its DNA. In general purely protein capsids render the phage more dense that lipid containing capsids (Coetzee et al., 1979).

ELECTRON MICROSCOPIC DETERMINATION OF MOLECULAR WEIGHT OF DNA. Visualisation and measurement of DNA molecules electron microscopically is a recognised method for determining their molecular weight. Due to variability in instrument magnification such determinations have to be performed relative to an internal standard of known molecular weight (Baltman et al., 1976, Mayer et al., 1973, Bernard et al, 1975 and Wensink and Brown, 1971). The absolute length of the molecule in question can be determined via an appropriate conversion factor.

Currently a factor of 1μm = 2,07 X 10^6 daltons (Clowes, 1972, Lang, 1970) and 1,508 kilobases = 1 X 10^6 daltons (Ohtsubo and Ohtsubo, 1977) are used.

PARTIAL DENATURATION MAPPING OF DNA. This involves the limited denaturation of DNA by heat or alkali. Regions which are rich in A-T base pairs have melting temperatures well below those of high G-C content (Funnell and Inman, 1979). Observation of such partially denatured molecules under the electron microscope shows the denatured regions as single stranded DNA (ss DNA) bubbles. For visualisation, the ss DNA is stabilised by formaldehyde which prevents reannealing (Compton and Sinsheimer, 1977). A particular DNA species will therefore display a characteristic partial denaturation pattern. This is best illustrated by integrating the patterns of individual molecules in a large population to give a denaturation map.

Studies involving analyses and comparisons of denaturation maps of DNA from many different sources have been performed. Such studies on Herpes Simplex virus (Delius and Clements, 1976) and human Cytomegalovirus (Kilpatrick and Haang, 1977) revealed, in both cases, two populations of molecules. These populations differed in that one population possessed a large inversion with respect to the other. The site(s) at which the inversion occurred could be pinpointed and the molecular weights of the inverted sequences could be

deduced. Partial denaturation analyses have been performed on many phage genomes allowing distinction between unique (λ, PR4 Mu) and permuted genomes (P22, T1, 5006M), (Davidson and Szybalski, 1971, Coetzee and Bekker, 1979; Bukhari and Taylor, 1975; Tye et al., 1974 a and b ; MacMattie and Gill, 1977 and Pretorius and Coetzee, 1979).

DNA DUPLEX FORMATION. This technique involves complete denaturation of DNA and subsequent renaturation. If performed on a mixture of two or more DNA species, those regions of the molecules in which base-pairing is conserved appear as double stranded segments, while regions of non-homology appear as single strand loops, bubbles or tails. (Compton and Sinsheimer, 1977). Thus one is able to compare directly sequences of DNA from different sources. The extent of homology or non-homology can be quantitated.

Physical mapping using such methods is hampered if the molecule under study is circular or permuted (Compton and Sinsheimer, 1977). A circular heteroduplex molecule with just a single strand bubble, loop or tail only gives information as to the extent of non-homology but not as to position. Determination of position requires that the heteroduplex has some other fixed physical characteristic of known position as a reference point.

Duplexes of permuted molecules are circular, with single strand protrusions corresponding to the terminal redundancy (Rhoades et al., 1968). This fact has been used to gain insight into the genomes of phages P22 (Rhoades et al., 1968) and 5006M (Pretorius and Coetzee, 1979).

RESTRICTION ENDONUCLEOTYTIC ANALYSES OF DNA. Restriction enzymes are endodeoxyribonucleases which recognise specific nucleotide sequences in double stranded DNA and cleave both strands (Nathans and Smith, 1975). In the bacterium, from which these enzymes are extracted they form part of the restriction-modification system (Arber, 1974). This system consists of a restriction enzyme which digests foreign DNA by cleaving it at a specific nucleotide sequence, and a modification enzyme which alters this site in the host and thus prevents autodigestion. Restriction endonucleases, when incubated with a DNA species, cleave the DNA molecules at their restriction site(s). These sites are present in the DNA due to chance occurrence of the appropriate nucleotide sequence. Therefore each DNA species has a characteristic digestion pattern consisting of fragments of varying sizes. The number of cleavage sites in a molecule for a given restriction enzyme depends on the size of the DNA molecule and the number of nucleotides forming the specific restriction site.

The characteristic digestion products can be analysed by gel electrophoresis. Choice of an appropriate gel system depends on the size of the fragments to be analysed. Polyacrylamide gels are used to analyse fragments of less than 10^6 daltons (Danna and Nathans, 1971). Fragments of 3-25 X 10^6 daltons are usually analysed on 0.3-0.8% agarose (Helling et al., 1974) and those between these two ranges require composite agarose-acrylamide gels (Sharp et. al., 1973).

Labeled DNA is visualised by autoradiography of the dried gel (Danna and Nathans, 1971) or a nitrocellulose filter onto which the DNA has been transferred (Southern, 1975). Alternatively the gel can be fractionated and counted.

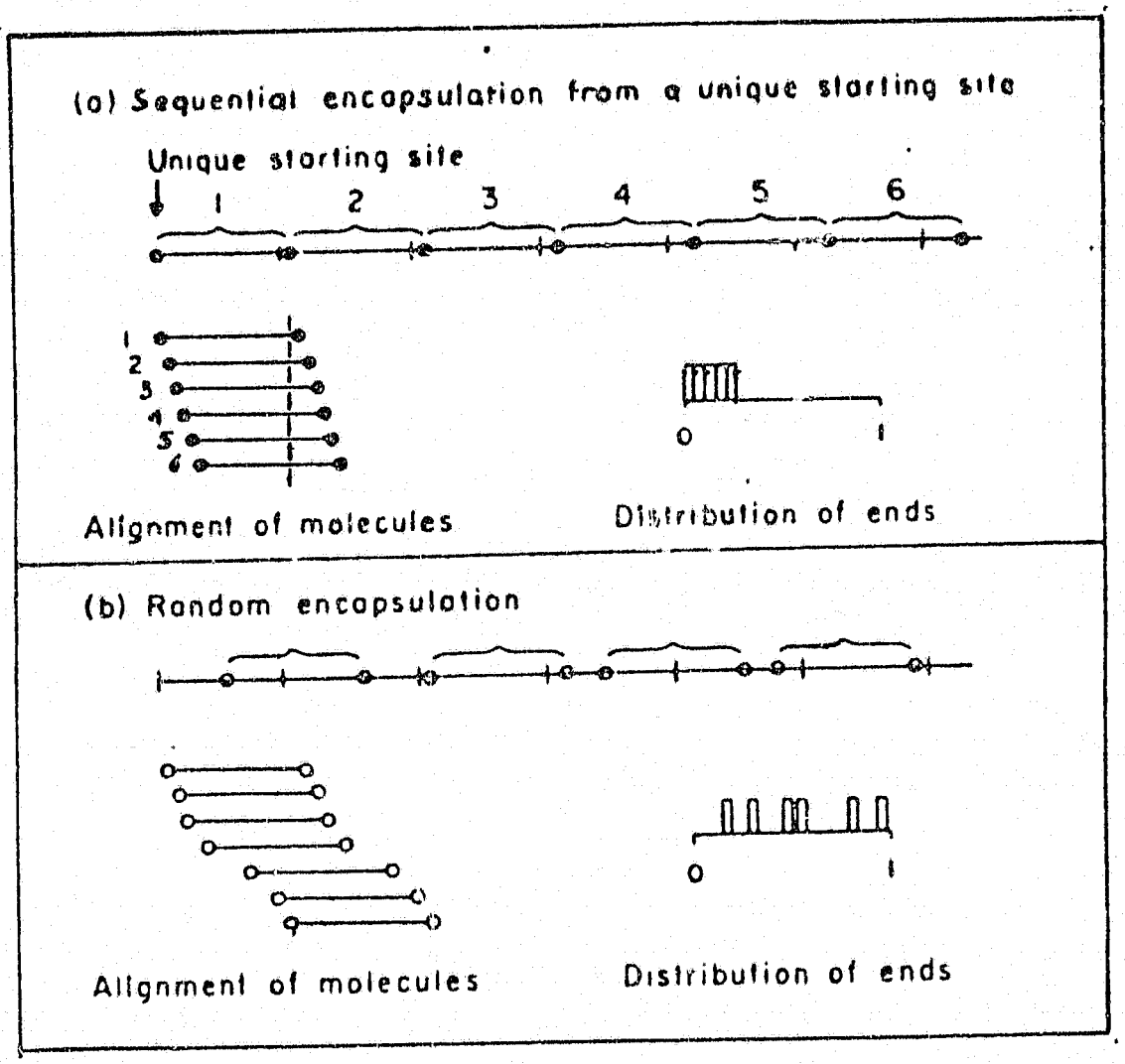
Staining of unlabeled DNA is usually carried out using ethidium bromide which intercalates into the DNA and can be excited by short or long wave ultraviolet light (Sharp et al., 1973).

Deletions, additions rearrangements and substitutions which may be undetectable by electron microscopy can be observed by restriction analysis. This has been the case for DNA from various SV40 strains (Nathans and Danna, 1972), polyoma virus (Fried et al., 1974), λ (Landy et al., 1974) and ϕ X 174 (Godson and Boyer, 1974).

VIRAL GENETICS

GENERALISED TRANSDUCING PHAGES are able to transduce any gene, regardless of its position on the host chromosome or on an extra- chromosomal element (Hayes, 1968). These phages have invariably been found to possess a double stranded DNA genome, with a terminal repetition. Moreover, all the genomes of generalised transducing phages studies to date have been found to be circularly permuted.

A model (Fig. 1) to explain the occurrence of permuted phage genomes has evolved through studies of coliphages T4 (Streisinger et al., 1964 and 1967) and P22 (Susskind and Botstein, 1978). The model presupposes the existence of concatemeric DNA which serves as an intermediate to DNA encapsulation. 'Headfuls' of more than a genome length are cut from the concatemer giving rise to phage heads containing circularly permuted terminally redundant DNA. Proof of such a model also requires the isolation of concatemeric DNA. Such proof has been found in P22 infected Salmonella (Botstein, 1968) and has been implicated in phage 5006M (Pretorius and Coetzee, 1979), the latter using the same techniques as the present study. Two alternatives to the model are presented (Fig. 1a and b) which could not initially be distinguished (Streisinger et al., 1964 and 1967). Electron microscopic (Tye et al., 1974 a and b) and restriction endonucleolytic (Jackson et al., 1978 a and b) and analysis showed that, in P22, the circular permutation extends over only 20 percent of the



(from Susskind and Botstein, 1978)

Fig. 1(a). Non-random sequential packaging of a phage concatemer from a unique site. Packaging of phage DNA results in encapsulation of a 'headful' of DNA, whose length is greater than one phage genome. This results in DNA with terminally redundant ends. Sequential packaging in one direction from a unique site results in a limited circular permutation of the genome. The maximum extent of the end point distribution indicates the maximum extent of the permutation.

Fig. 1(b). Random packaging from any site on the concatemer and proceeding in either direction. Such a packaging mechanism, of more than a set of phage genes, would result in a phage genome permuted over its entire length, indicated by the random end point distribution.

genome length. This finding shows that 'headful' packaging proceeds in a non-random, sequential manner. That packaging begins at a unique initiation site (pac) and proceeds in a unique direction can be inferred from the skew distribution of molecular ends (Fig. 1a) if one assumes that the packaging of the next headful is less likely than that of the previous headful (Tye et al., 1974 a and b).

Circularisation of the phage genome, on infection of a bacterial cell, appears to be a pre-requisite for concatemer formation and subsequent packaging. In the case of a phage having a unique (non-permuted) genome e.g. λ , circularisation is achieved by means of 'sticky ends' or cos sites (Nash et al., 1978). However, no 'sticky ends' are observed in phage whose DNA is packaged in excess of one genome length and therefore exhibit blunt ends (Tye et al., 1974 a and b). It has been observed that a P22 recombination deficient mutant (erf$^-$) will not propagate in a recA$^-$ host (Botstein and Matz, 1970). This has led to the theory that the linear phage genome circularises by a recombination event between the two terminal repeats. Phages lacking terminal repeats due to large insertions are defective, requiring double infection for propagation (Susskind and Botstein, 1978). It has been proposed (Susskind and Botstein, 1978) that the rolling circle model of DNA replication (Gilbert and Dressler, 1968) may form the link between the circularised monomeric genome and the concatemeric DNA intermediate.

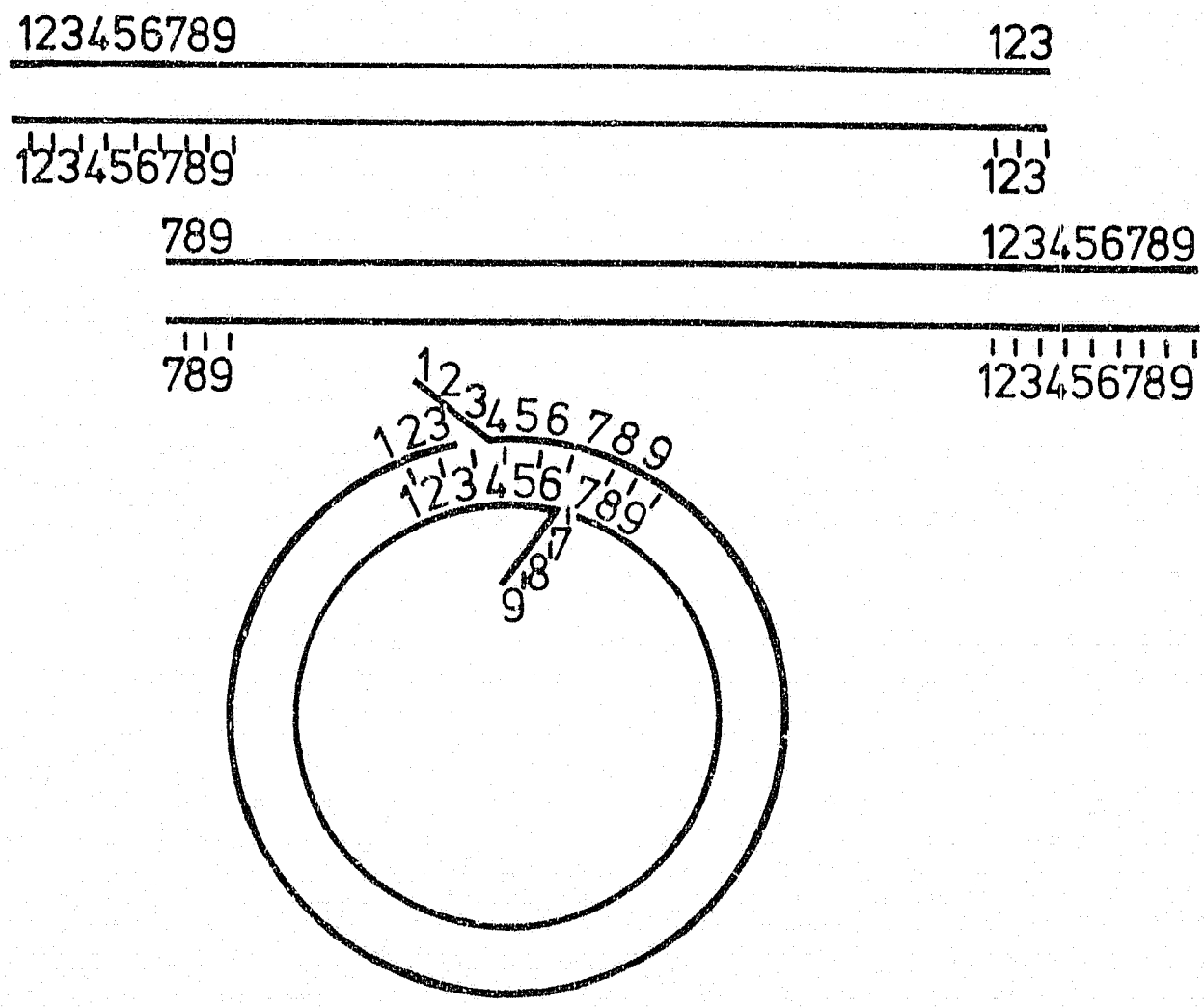


Fig. 2. 'Headful'-model prediction of structure expected in homoduplex experiments. The case illustrated could correspond to molecules 1 and 3 (Fig. 1a). Note single strand protrusions corresponding to terminal redundancy length. A duplex between molecules 1 and 4 (Fig. 1a) would result in a circle whose interprotrusion distance is increased by the length of the terminal repeat. Hence, inter-protrusion distance is determined by the terminal repeat and the concatemer length.

Homoduplex studies on such populations of permuted molecules reveal information as to the length of the terminal repeat (Rhoades et al., 1968). Duplex molecules circularise (Fig. 2) leaving the terminal repeats easily measurable. Measurement of the interprotrusion distance i.e. the shortest distance between the protrusions in a population of duplexes, mimics the end point distribution (Fig. 1a) and therefore reveals the extent of the permutation (Pretorius and Coetzee, 1979).

DNA packaging of P22 was also studied using EcoR1 restriction enzyme analysis (Jackson et al., 1978 a and b). Restriction analysis of non-permuted molecules yields fragments of different sizes which are all present in equimolar amounts. However, these workers discovered that the EcoR1 digest of the circularly permuted P22 genome reveals two bands of an unusual nature. The band migrating most slowly contains fragments that are quite heterogeneous in molecular weight, but due to the insensitivity of agarose gels at high molecular weight they are still observed as a discrete though somewhat diffuse band. The other unusual band was observed to be sharp but faint i.e. the DNA of the band was in less than molar amounts. The former is taken to represent those fragments having at one end the pac site and at the other an EcoR1 restriction site. This results in a majority of large fragments with a tail of small ones. The latter on the other hand represents those fragments with an EcoR1 site at one end and the permuted ends distal to the pac site at the other. This

results in a population of fragments of varying molecular weight, the smaller fragments of which are not observable giving rise to a faint but sharp band.

In order to determine the physical characteristics of the λ3 bacteriophage its buoyant density was determined. DNA extracted from an λ3 lysate was measured electron microscopically and by restriction enclonuclease analysis to obtain its molecular weight. Partial denaturation and denaturation-renaturation studies were performed and the results were analysed electron microsopically. Restriction analysis was used to study the λ3 genome further. This dissertation deals with these findings.

III : MATERIALS AND METHODS

All media, buffers and solutions are listed in the appendix.

BACTERIA AND PHAGES. A phage ∝3 lysate (Thomson and Woods, 1974) was obtained from the Genetics department, University of the Witwatersrand and is subsequently referred to as the original or stock isolate. Vibrio sp.2 (Thomson et al., 1972) was obtained from the same source. Proteus mirabilis PM5006 (Coetzee and Smit, 1970) and Proteus phage 5006M as well as its DNA were gifts of G.H.J. Pretorius (Microbiology department, University of Pretoria).

CULTIVATION OF PHAGE ∝3

Plate lysates. Phage was cultivated by the agar overlay technique (Thomson and Woods, 1974) using 10^5 p.f.u./plate and 0,1 ml of log. phase Vibrio sp.2 ($O.D._{550}$ = 0,8). Soft agar (LSSA) was scraped off Luria salt agar plates (LSA) which had been incubated overnight at 30°C. Ten millilitres of 0,4M saline per plate was added and the mixture was left at 4°C for several hours. The agar and cell debris were pelleted in a JA-10 rotor in a Beckman J-21 centrifuge at 10 000 r.p.m. for 1 hour.

Liquid lysates. Vibrio sp.2 was grown in Luria salt broth (LSB) at 37°C, with aeration to $O.D._{550}$ = 0,2. Phage was added at a m.o.i. of 0,5 and the temperature reduced to 30°C. $O.D._{550}$ was monitored until lysis was observed (approximately 6 hours). Cell debris was pelleted in a Beckman JA-10 rotor at 10 000 r.p.m. for 30 minutes.

High titres were grown, both from the stock α3 lysate and from 6 single plaques. These were stored above chloroform with no loss of infectivity.

PURIFICATION OF PHAGE α3

Lysates were concentrated using polyethylene glycol 6000 (PEG) (Yamamoto and Alberts, 1970). The optimal PEG concentration was determined at 0,5M NaCl (final concentration) by varying PEG concentrations from 5 to 20 percent. PEG was thoroughly dissolved and the lysate left at 4°C overnight. The lysate was spun in Beckman rotor JA-10 at 8 000 r.p.m. for 10 minutes and pellets resuspended in 0,4M saline at 4°C for several hours (concentrating phage 100 times). PEG-precipitated phage was cleared by spinning at 10 000 r.p.m. for 45 minutes in a Sorval SS-4 bench centrifuge and an S34 rotor. The phage was pelleted by spinning for 2 hours at 35 000g. Phage pellets were resuspended in 0,4M saline overnight at 4°C. After a clarifying spin (as above) CsCl was added to give ρ = 1,5 and η = 1,38, where ρ is the density and η is the refractive index (Bruner and Vinograd, 1965). The gradient was formed by spinning for 48 hours in a SW-50.1 rotor at 33 000 r.p.m. in a Beckman L2-65B ultracentrifuge. The phage band was removed and dialysed overnight against TE buffer to remove CsCl.

BUOYANT DENSITY OF PHAGE α3

This was determined according to Pretorius and Coetzee (1979), using the Proteus phage 5006M as standard. This method involves mixing the two phages (α3 and 5006M) to give equal titres of 10^7 p.f.u./ml. CsCl was added to give ρ = 1,5.

A gradient was formed (see phage purification) and fractionated. Refractive indices and spot titres (of both phages) were determined for each fraction. Appropriate fractions were subsequently titred in detail and in duplicate. Phage λ3 was plated on Vibrio sp.2 and 5006M was plated on P. mirabilis PM5006.

DNA EXTRACTION

DNA was extracted from purified λ3 phage using several phenol extractions followed by extensive dialysis against TE buffer (Mandell and Hershey, 1960). Plasmid Col E1 DNA was a gift from G.H.J. Pretorius.

ELECTRON MICROSCOPY OF PHAGE λ3 DNA

Electron microscopy was performed on DNA extracted from purified high titres of the original stock of λ phage. Phage DNA was visualised using the method of Coetzee and Pretorius, (1979). Briefly, phage DNA was diluted to a final concentration of 0,2 μg/ml in 100 μl of droplet solution (appendix). An aliquot of 1-5 μl of 0,2 percent benzydimethylammonium chloride (BAC) in 100 percent formamide was added per 100 μl droplet solution. Droplets were formed on parafilm and left covered for 10 minutes. A 300 mesh, carbon coated grid was applied to each droplet. The grids were floated face down on redistilled water (25 seconds for visualising single stranded DNA and up to several minutes for double strands). They were then submerged for 20 seconds in a freshly made 1:300 dilution of 50mM uranylacetate 50mM HCl in 90 percent ethanol, and washed in 90 percent ethanol. Grids were rotary shadowed in an Edwards 12 E 12 evaporator using platinum or gold-palladium. DNA was visualised in a Philips EM 301 electron microscope.

Molecular weight determination. The molecular weight of the phage DNA was calculated using the Col E1 plasmid (molecular weight is 4,2 x 10^6 daltons; Bazaral and Helinski, 1968) as internal standard. Phage DNA molecules were measured and compared to Col E1 molecules present in the same field of the electron microscope.

Partial denaturation mapping. Partial denaturation mapping of the phage genome involved heating of the phage DNA for 10 minutes at 58°C in denaturation solution (appendix). Dilution into droplet solution (final DNA concentration was 0,2 µg/ml) was followed by the electron microsopy procedures described above.

Duplex formation. Duplexes were performed as described (Pretorius and Coetzee, 1979). Briefly, this involved diluting the DNA to a final concentration of 6 µg/ml in 90 percent formamide and subsequent heating at 50°C for 10 minutes. After addition of 0,7 volumes of 5 x SSC, the DNA was incubated at 30°C for 15 minutes. The DNA was then diluted with 0,6 volumes of a mixture of 5 x SSC and formamide (in the starting ratio of 3:5). The resultant solution was reincubated at 30°C for several hours. This was mixed with formaldehyde in the ratio 7:1 respectively. An aliquot was added to droplet solution to give a final DNA concentration of 0,2 µg/ml and processed for the electron microscope as previously described.

RESTRICTION ANALYSIS

Restriction analysis was carried out using DNA from purified high titres grown from both the original λ3 stock and from 6 single plaque isolates. Restriction endonucleases were Eco R1, Bam H1 and Hind III (Boehringer, Mannheim). The

following method was used. Restriction was carried out in a total volume of 50 µl. DNA was diluted in the appropriate restriction buffer (appendix), to a final concentration of 12 µg/ml (0,6 µg DNA per restriction). Restriction enzyme (1-2 µl) was added and the restriction carried out at 37°C for 2 hours. Prior to loading of the gel, 5 µl of a 75 percent sucrose, 0,02 percent bomophenol blue solution was added to each restriction.

Agarose (0,75 percent) was boiled in 90 ml redistilled water. Electrophoresis buffer (10 ml of 10 times concentrated) was added. The agarose was poured to form a horizontal gel of dimensions 140 x 190 x 3mm., having 10 wells each able to hold 50 µl. Two percent agarose in electrophoresis buffer was used as wicks (McDonell et al., 1977). Gels were run for 8-10 hours at 60 volts.

Prior to photography the were stained in electrophoresis buffer containing 1 µg/ml ethidium bromide at 4°C for 1 hour. Illumination was from below, using General Electric long wave ultraviolet lamps. Photographs were taken with a Polaroid camera EE66 with a type 665 film or a Pentax SPF with an Ilford FP4 film. The cameras were fitted with standard ultraviolet and red filters.

Negatives were scanned using the film scanning accessory of a Varian Cary 210 spectrophotometer.

IV : RESULTS

CULTIVATION AND PURIFICATION OF PHAGE α3

Phage lysates were obtained from the original stock of α3 and from 6 single plaques. Plate and liquid lysates gave titres of 10^{10} p.f.u./ml. It was found that 14 percent PEG was optimal for phage α3 precipitation at 0,5M NaCl (Fig. 3). PEG-precipitated lysates gave titres of about 10^{12} p.f.u./ml during bulk purification. Phage extracted from CsCl gradients gave titres in the order of 10^{13} p.f.u./ml.

BUOYANT DENSITY OF PHAGE α3

The CsCl gradient was fractionated into 38 fractions of approximately 0,13 ml each. The observed refractive index, n, was converted to density, ρ, using the relation $\rho^{25} = 10{,}2402\, n_{D25} - 12{,}6483$ (Bruner and Vinograd, 1965). Spot titres against Vibrio sp.2 and Proteus mirabilis PM5006, indicated that phage α3 was present in fractions 13-22 and 5006M in fractions 16-23. Accurate titres of both phages (from the appropriate fractions) were converted to a percentage of total phage recovered and this was plotted together with the buoyant density (Fig. 4).

Centres of gravity (in units of fraction number) for the peaks (Fig. 4) were calculated as follows:
F(α3) = 18,92 and F(5006M) = 20,74. The density-fraction number relationship (Fig. 4) is:

$\rho = -0{,}008F + 1{,}662$ g.cm.$^{-3}$ 1

From Fig 4 ρ(5006M) = 1,496 g.cm.$^{-3}$.

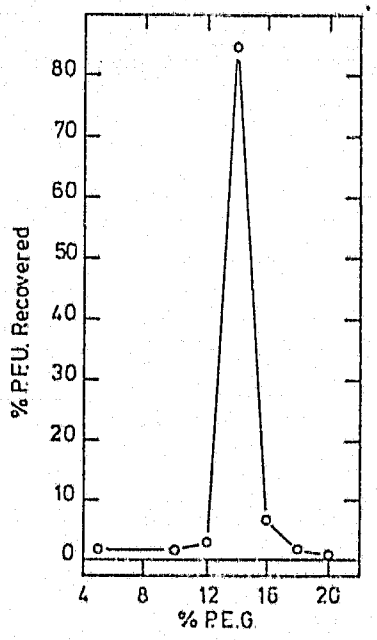


Fig. 3. Precipitation of phage by polyethylene glycol 6000 (PEG). One hundred ml. aliquots of α3 lysate (10^9 p.f.u./ml) were made 0,5M with NaCl and PEG was added at varying concentrations. After the PEG was dissolved, the lysate was stored at 4°C overnight. Precipitated phage was pelleted at 10 000 r.p.m. for 5 minutes in a Sorval SS-4 bench centrifuge rotor S-34 and resuspended overnight in 1,0 ml saline. After a clarifying spin, this was titred and percent phage at each PEG concentration was calculated.

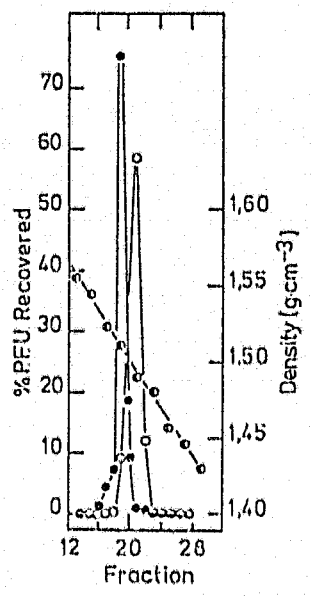


Fig. 4. Percent plaque forming units recovered and density vs. fraction number. Phages α3 and 5006M were centrifuged in CsCl to form a gradient. Fractions were titrated and results are presented as percent phage recovered fromm relevant fractions. The buoyant density was calculated from the refractive index of each fraction. o-o phage α3, o-o phage 5006M and o-o density.

However, ρ (5006M) is known to be 1,491 g.cm.$^{-3}$ (Pretorius and Coetzee, 1979), therefore the correction factor $\Delta\rho$ is - 0,005 g.cm.$^{-3}$. From equation 1 ρ (α 3) = 1,511 g.cm.$^{-3}$. Application of the correction factor gives ρ (α 3) = 1,506 g.cm.$^{-3}$.

DNA EXTRACTION

DNA was extracted from the α3 stock lysate and from the 6 single plaque isolates at a concentration of about 60 µg/ml.

ELECTRON MICROSCOPY OF PHAGE DNA

Molecular weight determination. Forty α3 DNA molecules were measured together with a similar number of Col E1 molecules. Micrographs were taken at an electron microscope magnification of 5900 (Fig. 5). From the distribution of phage lengths with respect to Col E1 length (Fig. 6) it was calculated that the length ratio of α3 DNA : Col E1 is 8,2 ± 0,2.

Assuming that Col E1 is 6,34 kb (Ohtsubo and Ohtsubo, 1977), it follows that the genome size of α3 phage is 6,34 x 8,2 kb, which is 51,98 ± 1,29 kb. The conversion of kilobases to daltons follows the relation, 1,508 kb equals 1 x 10^6 daltons (Ohtsubo and Ohtsubo, 1977). Using this conversion factor, one obtains an α3 genome molecular weight of 34,66 ± 1,95md. This may be compared to the values obtained by restriction analyses (see p30).

Partial denaturation mapping. Partial denaturation studies were carried out on 63 molecules of α3 DNA (from the original α3 stock) (Fig. 7). After digitisation, the molecules were computer-simulated (Fig. 8). Computer alignment of

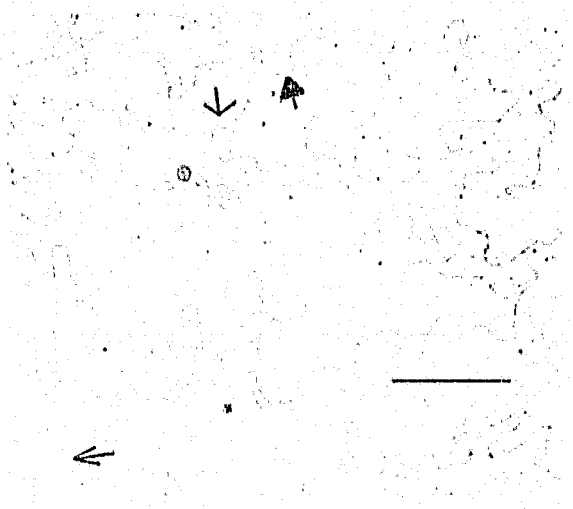


Fig. 5. Electron micrograph of an ∝3 genome and ColE1 plasmids. Phage ∝3 genome lengths were measured with respect of ColE1 present in the same field. Bar indicates 1 um. Phage ∝3 ← ; plasmid ColE1 ←.

Fig. 6. Distribution of ∝3 genome lengths with respect to the length of plasmid ColE1. The length of the ∝3 genome was found to be 8,2 ± 0,2 times the length of plasmid ColE1. This was converted to kilobases (51,98 ± 1,29 kb) and subsequently to molecular weight (34,66 ± 1,95 Md).

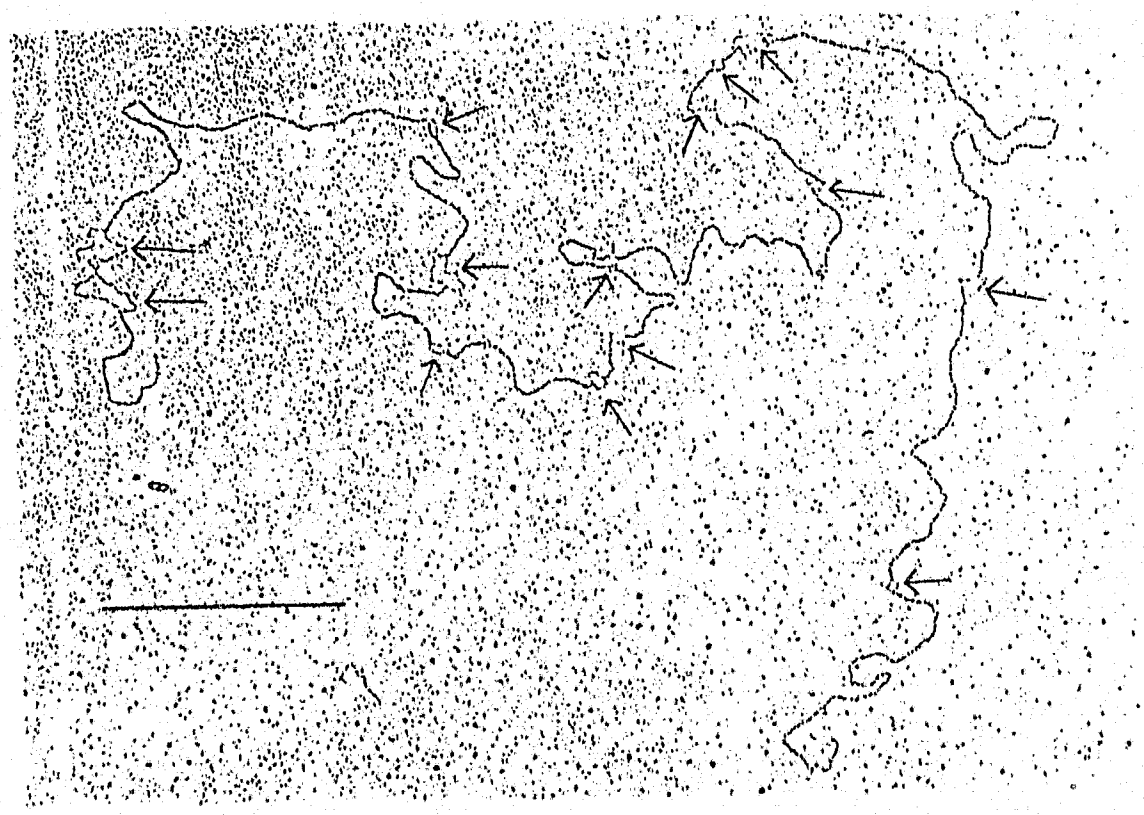


Fig. 7. Partially denatured λ3 genome. Phage DNA was heated to 58°C for 10 minutes in denaturation solution. The DNA was then diluted into droplet solution (final DNA concentration of 0,2 µg/ml). DNA was adsorbed, from droplet onto carbon coated grids and rotary shadowed with Pt/Pd. Bar indicates 1 µm. Denatured regions are indicated.

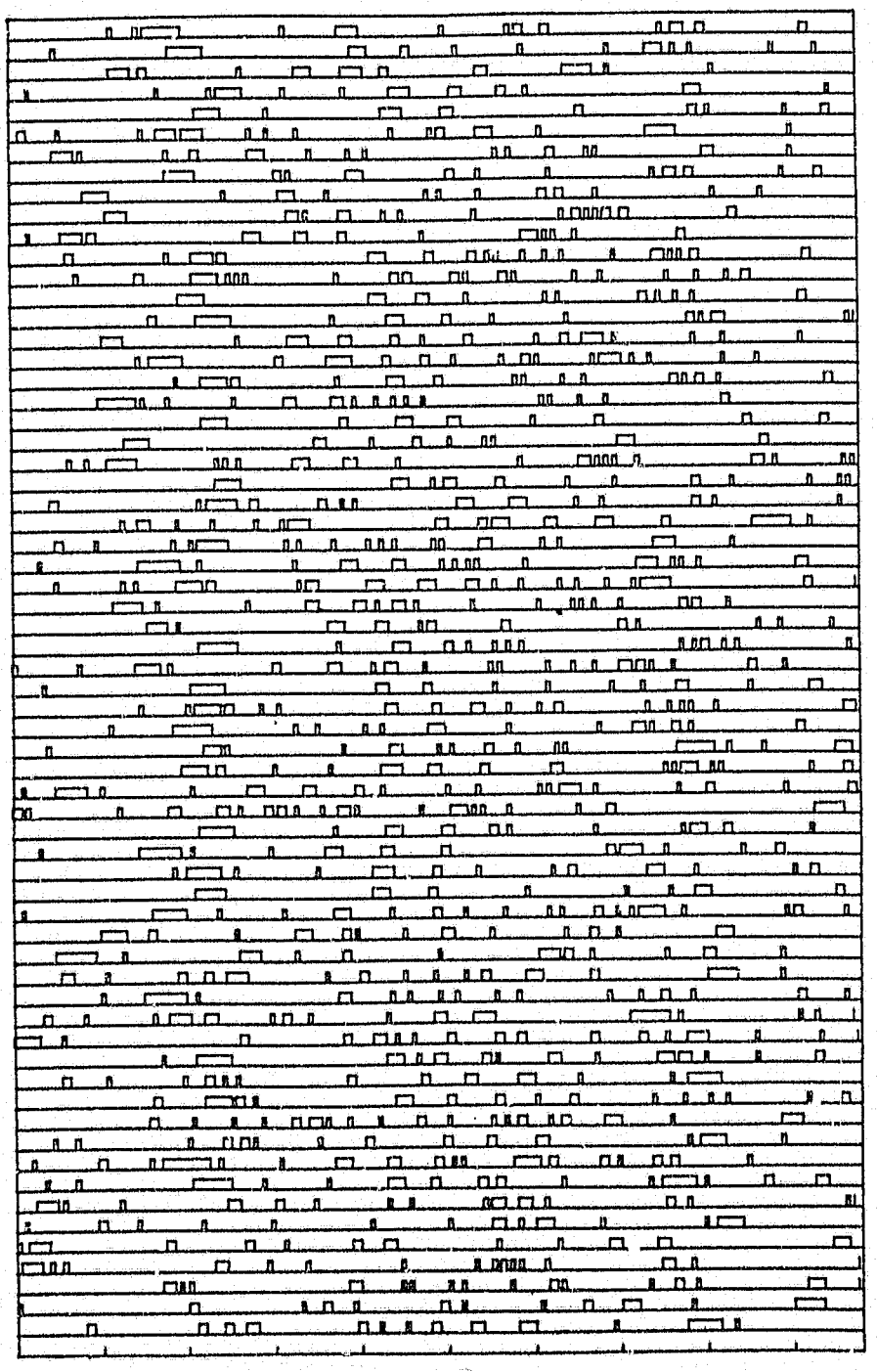


Fig. 8. Computer simulations of partially denatured phage α3 DNA. Sixty three partially denatured α3 molecules were digitised and computer simulated. Denatured regions are represented by 'bubbles'.

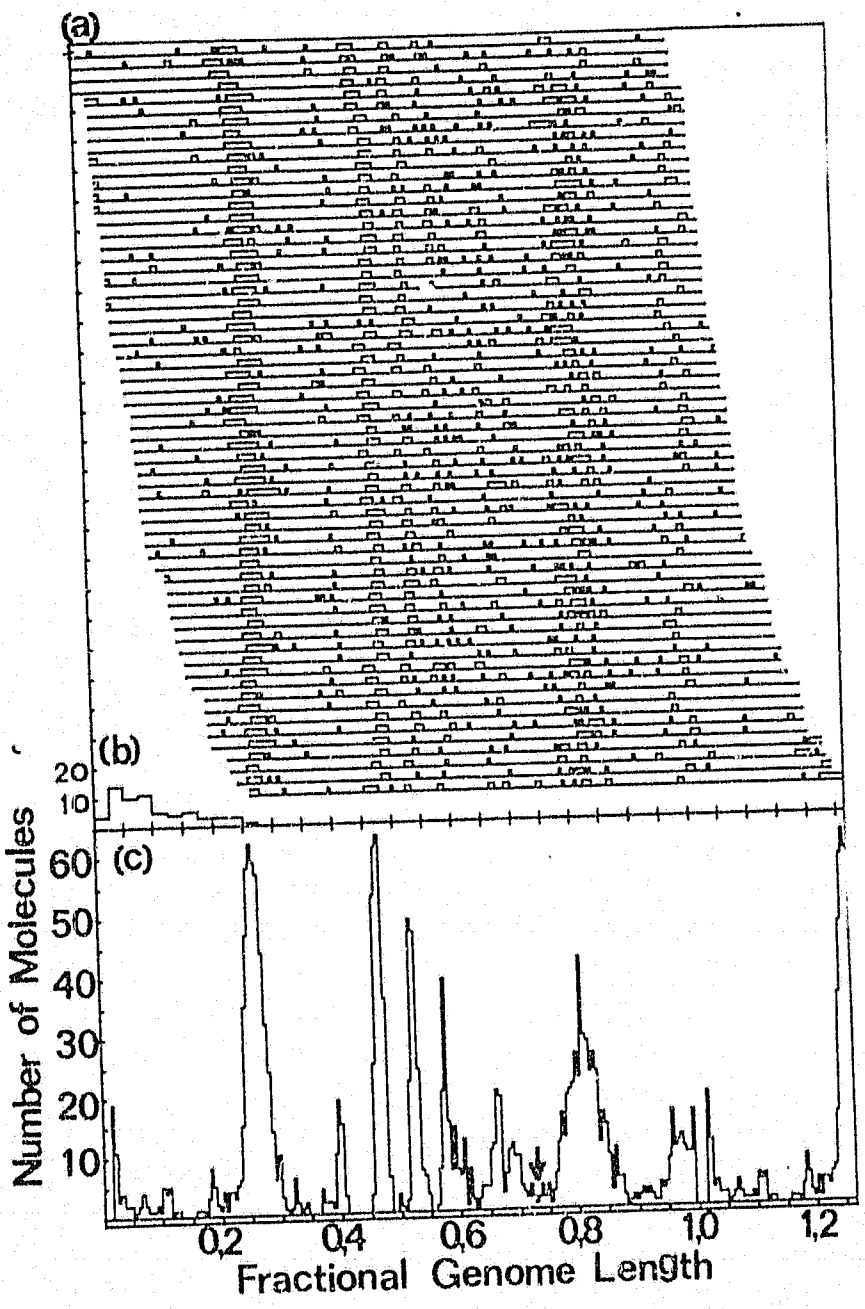


Fig. 9(a) Computer alignment of 63 partially denatured λ3 genomes. The molecules were aligned in order of increasing percent denaturation and the terminal repeat was omitted from the alignment. Note that denatured regions are not uniquely located in all the molecules, suggesting permutation of the genome.
Fig. 9(b) Distribution of molecular ends. This was obtained by integrating the end point locations of Fig. 9(a). Note that a skew distribution is generated, and extends over 27 percent of the genome. The latter indicates that the λ3 genome is circularly permuted over this percentage of its length. 'Skewness' signifies sequentially oriented packaging as from a unique site (pac) at the axis origin and proceeding towards the right of the figure.
Fig. 9(c). Partial denaturation map. This was derived by integrating the molecular information (Fig. 9a) with respect to the occurence of denatured regions. Note that the first six peaks from the left (at positions less than 0,75 of the genome length) are very sharp indicating excellent correspondence of molecular information. The last two peaks (at positions between 0,75 and 1,0 of the genome length) are, however, less well resolved and indicate poorer information correspondence in this region of the molecule.

the molecules (Fig. 9a) shows that ϕX3 DNA is non-randomly circularly permuted. The distribution of molecular ends (Fig. 9b) was derived from Fig. 9a, and shows that the permutation of ϕX3 DNA extends over 27 percent of its length. The denaturation map (Fig. 9c) was derived by integrating the information, presented in Fig. 9a, with respect to the occurrence of denatured regions.

The first six major peaks from the left (Fig. 9c) are very sharp, indicating excellent correspondence of molecular information between individual molecules (Fig. 9a). The remaining two major peaks do not display this characteristic, suggesting less precise correspondence of molecular information in this region of the molecule.

Duplex Formation. Duplex molecules, resulting from complete denaturation of ϕX3 DNA and subsequent renaturation were seen to circularise (Fig. 10a and b). Circularisation is a result of circular permutation (Tye et al., 1974 a and b; Pretorius and Coetzee, 1979). Circular molecules also possess two single strand DNA protrusions as a direct consequence of terminal redundancy in permuted molecules (Tye et al., 1974 a and b). Forty ϕX3 duplexes were photographed and two classes of molecules were observed. One class of molecules (Fig 10a) possesses the expected two single strand protrusions, whilst the other, in addition to the expected protrusions possesses a single strand loop (Fig. 10b).

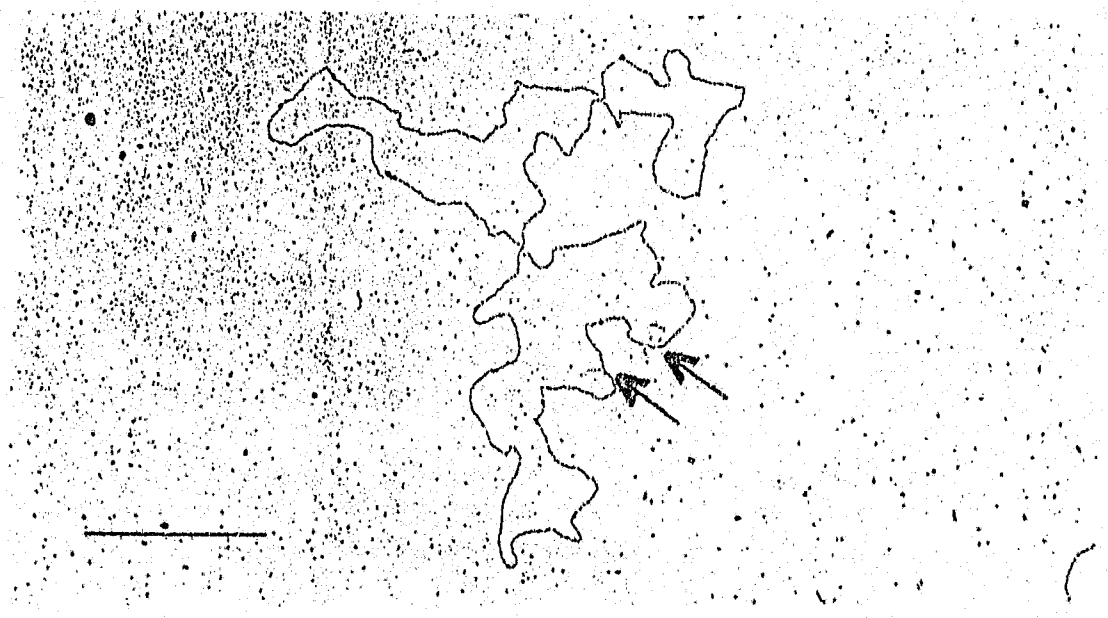


FIG 10(a). Homoduplex of λ3. Denaturation-renaturation of λ3 DNA was found to result in circularisation. Two single strand protrusions are observed (arrows), which are due to terminal redundancy in permuted molecules. Single strand protrusions were found to be 2,4 $\pm$ 0,8 percent of the phage genome, as determined from 40 molecules. Bar represents 1 μm.

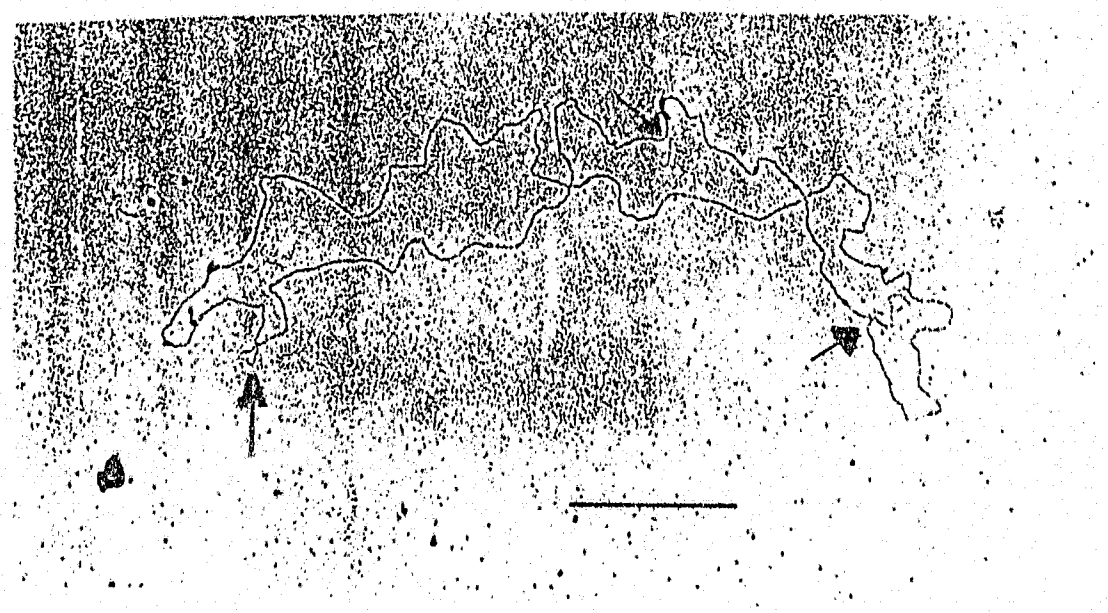


Fig. 10(b). Heteroduplex of λ3. A second class of circular molecules was observed from the DNA of the λ3 stock lysate. These possessed, in addition to the two single strand protrusions, a single strand loop (arrow). This suggests a dual phage population, namely one with an insertion or deletion in its genome with respect to the other. The single strand loop was observed and measured on 12 molecules as 2,0 percent of the genome length. Single strand protrusions ⟵.

The first class of duplex molecules (Fig. 10a) represents the typical homoduplexes one would expect. The second class, however, suggests two molecular populations, which on reannealing effectively form heteroduplexes (Fig. 10b). The proposed two molecular populations have extensive areas of homology. In fact, measurements of heteroduplexes reveal the area of non-homology (the single strand loop) to be only 2,0 ± 0,4 percent of the total molecular length. This appears to indicate an insertion or deletion of 2,0 ± 0,4 percent in one molecular population with respect to the other.

Measurements of the single strand protrusions, which are in effect the terminal repeat (Tye et al., 1974 a and b) yield average lengths of 2,4 ± 0,8 percent for the homoduplexes and 2,4 ± 1,1 percent for the heteroduplexes. Inter-protrusion distances (the shortest distance between protrusions) of 40 molecules are presented graphically (Fig. 11a). These are a consequence of the extent of permutation (Pretorius and Coetzee, 1979). Protrusion-loop distances, measured from 12 molecules are presented (Fig. 11b). It will be noted that inter-protrusion distances range from 0 to 30 percent of the genome, whereas protrusion-loop distances are never less than 20 percent.

RESTRICTION ANALYSIS

Restriction patterns (Fig. 12) of the single plaque isolates show no observable difference when Eco R1 (wells 1 and 2) or Bam H1 (wells 3 and 4) are used. However, using Hind III one observes a difference between the single plaque isolates. This difference shows itself as an extra band (Hind α) which is present in one single plaque isolate (population α 3.1)

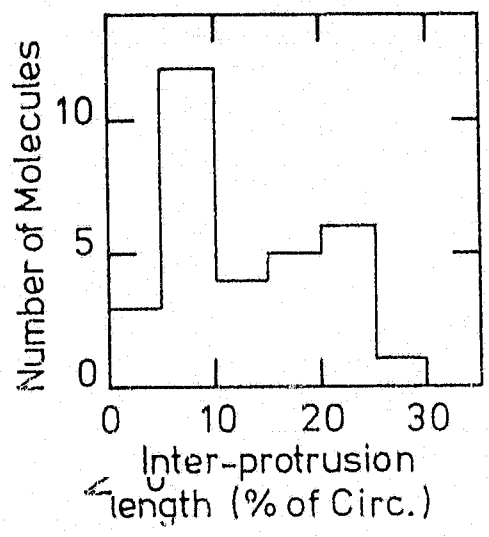


Fig. 11(a). Distribution of inter-protrusion distances. These were measured in 40 molecules and plotted. Such a distribution should mimic that of the end point distribution of Fig. 9b (Pretorius and Coetzee, 1979), having a cut off point at 27 percent. However, an upward trend is observed at 20-25 percent, and this is explained in terms of the erroneous inclusion of ill-resolved heteroduplexes.

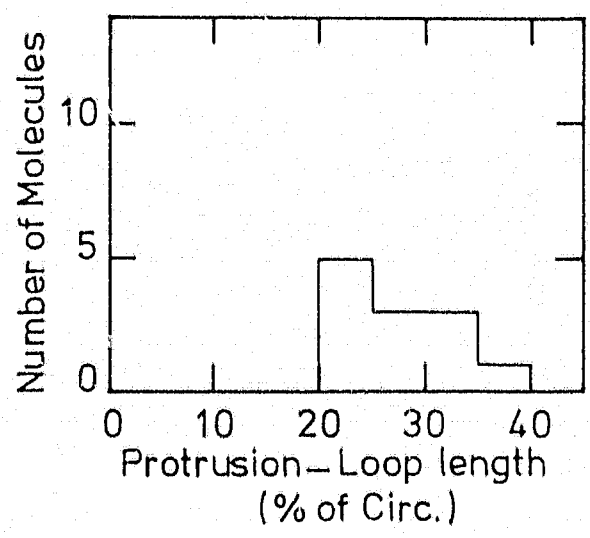


Fig. 11(b). Distribution of Protrusion-Loop distances. The distance between the single strand loop and the nearest protrusion was measured. Note that the minimum distance observed is 20-25 percent of the total molecule length. Also note, that the distribution decreases towards increasing percentages. This implies that the relative insertion has occurred 20-25 percent from that end of the molecule, distal to the pac site. A location of 20-25 percent from the pac site would have led to a distribution exhibiting a maximum at the insertion/deletion site (as observed) but a decrease towards the axes origin.

but not in the other (population α 3 2). Of the 6 single plaque isolates screened, 4 exhibited band α (α 3.1) and 2 did not (α 3.2). The original α3 stock also possesses Hind α .

Using a partial Eco R1 digest of phage 5006M DNA as molecular weight standards (Pretorius, personal communication), the molecular weights of the Hind III restriction fragments were determined (Fig. 10) as follows: Hind A/B (8,2Md), Hind α (7,6Md), Hind C (6,4Md), Hind D (4,5Md), Hind E (3,7Md) and Hind F (2,9Md). These give a total fragment size of 33,3Md for α3.1 and 33,9Md for α3.2.

The Hind III restriction pattern of α3 DNA shows one relatively less intense band: Hind C (Fig. 12). The origin of this band may be traced to the circular permutation of genomes (Jackson et al., 1978 a and b).

The relatively low intensity of band Hind C can be clearly seen on the spectrophotometric scan of Hind III restriction of α3.1, α3.2 and the original α3 lysate (Fig. 13).

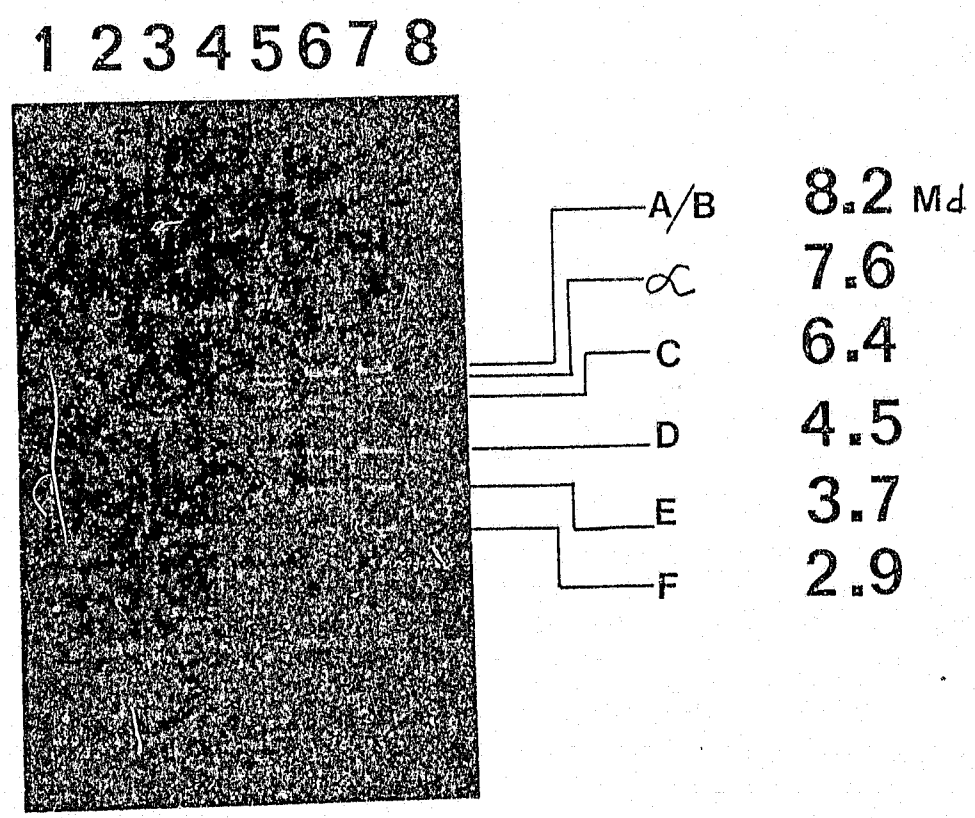


Fig. 12. Comparison of restriction patterns of DNA from phage ∝3 lysates. Eco R1 (lanes 1 and 2) and and Bam H1 (lanes 3 and 4) restrictions of ∝3.1 and ∝3.2 exhibit no difference between two single plaque isolates. Hind III restriction (lanes 5 and 6) exhibits band ∝ in one of the single plaque isolates (∝3,1, lane 5) but not in the other (∝3.2, lane 6). Band ∝ is also observed in the Hind III pattern of the original ∝3 lysate (lane 7). Note the relatively faint band Hind C. Molecular weights (Md) were derived from a calibration curve based on phage λ fragment weights to which the Eco R1 partial digest of 5006M DNA (lane 8) had been related.

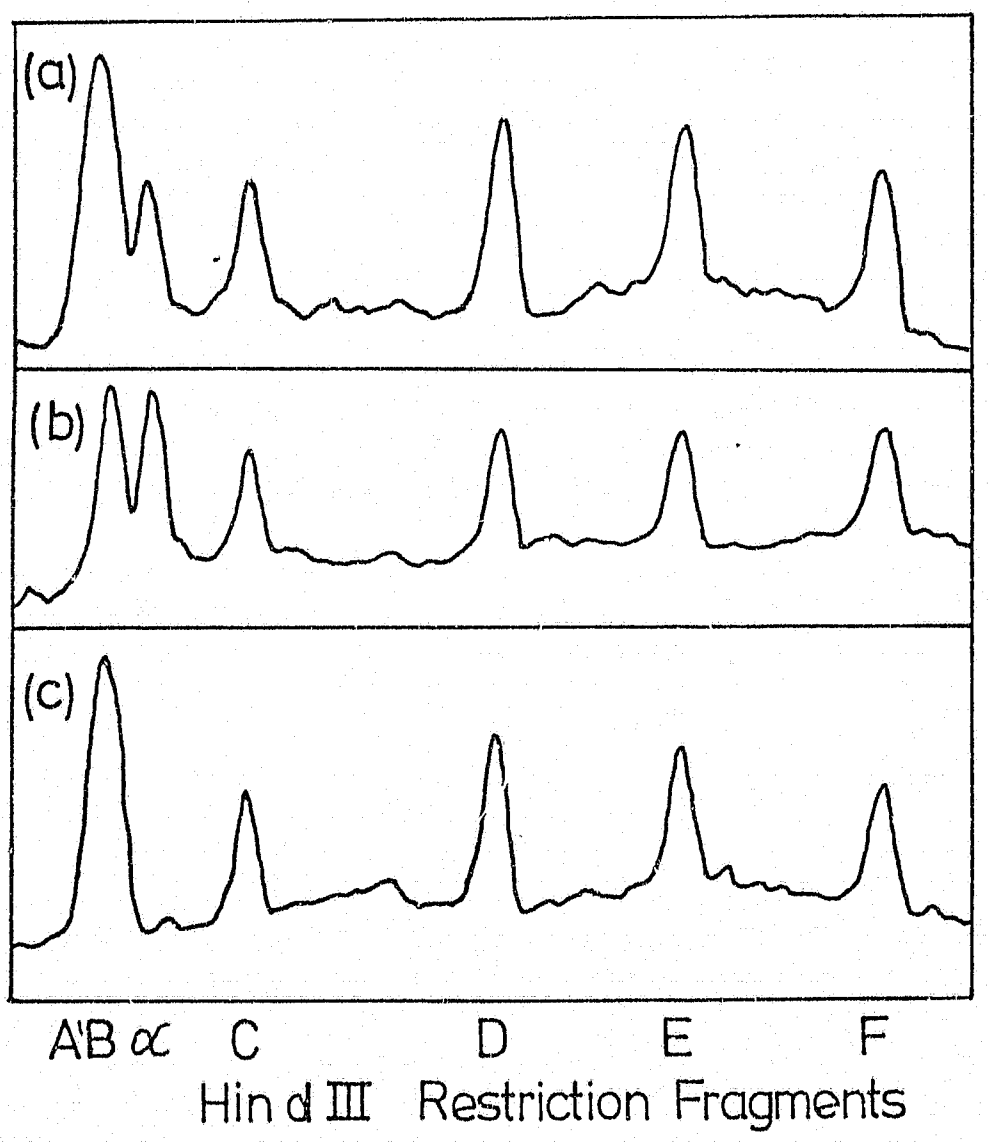


Fig. 13. Spectrophotometric scan of the Hind III restriction patterns of (a) original α3 stock (b) population α3.1, (with the relative deletion) and (c) population α 3.2 (with the relative insertion). Note that band A/B (population α3.2) is resolved into two bands (population α3.1) and that both populations are present in the original stock. Also note the less intense band Hind C in all cases.

V : DISCUSSION

Considering the ϕ3 buoyant density (1,506g. cm.$^{-3}$.), genome molecular weight (34,66 x 10^6) and G.C. content (Thomson and Woods, 1974) it can be concluded that phage ϕ3 has a protein capsid (Weigle and Meselson, 1959). Chloroform sensitivity is usually associated with a lipid containing capsid (e.g. Bradley and Rutherford, 1975; Coetzee et al., 1979). Therefore , an alternative basis for the reported ϕ3 chloroform sensitivity (Thomson and Woods, 1974) may be indicated.

Partial denaturation studies (Fig. 9a and b) reveal that the phage ϕ3 genome is non-randomly circularly permuted over 27 percent of its length. This is shown by the distribution of molecular ends (Fig. 9a and b). Non-random circular permutation is attributed to the 'headful' mode of packaging (Tye et al., 1974 a and b). In phage ϕ3 packaging starts at the pac site (at the abscissa origin) and proceeds from left to right (Fig. 9c). Therefore the 'headful' packaging hypothesis can now be extended to include the Vibrio phage ϕ3.

The length of the concatemer can be estimated by denaturation-renaturation studies, which result in circular molecules, (Rhoades et al., 1968). Circularisation is represented diagramatically (Fig. 2). Such circles were observed for phage ϕ3 (Fig. 10a). These circles show 2 single strand protrusions whose lengths can be equated with that of the terminal repeat (Fig. 2). Phage ϕ3 has a terminal repeat of 2,4 ± 0,8 percent of its DNA length. This fact, together

with the length of the maximum permutation of 27 percent facilitates calculation of a concatemer length of 11 ± 3 'headful' equivalents. Such a concatemer length is in agreement with those proposed for P22 (Tye et al., 1974 a and b) and for 5006M (Pretorius and Coetzee, 1979) namely 10 and 12 ± 3 respectively. In vivo formation of a concatemer requires prior circularisation of the phage genome. In phage ∝3 this was achieved via a recombination event between the terminal repeats, as is the case in P22 (Susskind and Botstein, 1978) and 5006M (Pretorius and Coetzee, 1979).

Denaturation-renaturation of ∝3 molecules revealed two populations of circles : the expected circles with two single strand protrusions (Fig. 10a) and those having a single strand loop structure in addition to the two usual protrusions (Fig. 10b). The latter is taken to indicate that the phage ∝3 lysate used in these experiments consists of two phage populations, one with an insertion or deletion of 2,0 ± 0,4 percent of the genome length with respect to the other.

As phage ∝3 is packaged by a 'headful' mechanism a relative insertion or deletion (as above) will not affect the molecular weight determined electron microscopically and will exert little or no influence on the phage buoyant density. In contrast, however, such an insertion or deletion should affect the length of the terminal repeat (Susskind and Botstein, 1978). One would, therefore, expect the ∝3 heteroduplexes (Fig. 10b) to exhibit single strand protrusions whose lengths differ by the length of the single strand loop. In view of

the large statistical variation on the present measurements and also on similar such measurements (Pretorius and Coetzee, 1980) the observed protrusion length of 2,4 percent is not at variance with the permissible difference of 1,9 percent. Another observable consequence of a 2 percent relative insertion or deletion is seen in Fig. 11 a and b. The distribution of inter-protrusion distances (Fig. 11a) should mimic that observed for molecular end points (Fig. 9b) (Pretorius and Coetzee, 1979). This distribution (Fig. 11a), however, displays an upward trend at 20-25 percent and continues past the expected cut off point of 27 percent that Fig. 9b suggests as limiting. This unexpected feature can be explained if some of the contributing molecules with inter-protrusion distances of 20-25 percent and upwards were in fact heteroduplexes. Such erroneous inclusion of heteroduplexes can result from molecules with poorly resolved or broken loops and loss of one protrusion. The latter feature is observed quite frequently.

The protrusion-loop distance distribution (Fig. 11b) runs from 20-25 percent upwards, clearly indicating that the genome is altered at ~20-25 percent from the terminus distal to the pac site. More evidence to this effect can be seen from the partial denaturation map (Fig. 9c). The two less sharp peaks at the right-hand side of the map may reflect the insertion or deletion in one sub-population of molecules with respect to the other. The denaturation map, therefore, also suggests that the insertion or deletion site is 20-25 percent (minimum protrusion-loop distance) from the right-hand end of the molecule (i.e. 75-80 percent from the pac site). The proposed site is indicated by an arrow (Fig. 9c).

Confirmation for the existence of a mixture of molecular populations in the λ3 lysate used in the initial studies, results from the restriction analysis (Fig. 12) which displays the absence of the Hind λ fragment from one single plaque DNA population (λ 3.2) as compared to the other (λ 3.1). It appears that the double band A/B (λ 3.2) is resolved into two bands A and λ (λ 3.1). The molecular weight difference between A and λ is 0,6 x 10^6, in agreement with the size of the deletion observed electron microscopically (0,7 $\pm$ 0,2 x 10^6). Hence the λ3.1 genome exhibits a deletion relative to the λ3.2 genome. Densitometric scanning of the Hind III restriction pattern (Fig. 13) of λ3.1, λ3.2 and λ3 (original lysate) indicates that in the latter the λ3.1 : λ3.2 ratio is 1,25.

Failure to demonstrate a difference with Eco R1 and Bam H1 may be explained if the relative deletion is located on a fragment much larger than Hind A/B in these digests. Such a small difference (0,7 $\pm$ 0,2 x 10^6) is not easily resolved on 0,75% agarose gels for large fragments.

It is therefore concluded that the λ3 stock used in these studies consists of a mixture of two phage populations whilst the single plaque isolates are homogeneous. This fact is interesting as no suggestion of a dual phage population has been observed during standard genetic manipulation of this phage-host system. Further, a practical application of this homogeneity presents itself. Compton and Sinsheimer (1977) have pointed out the difficulties of correlating the physical map (partial denaturation) with the genetic map in molecules

which are not unique and which do not have distinguishable ends. Phage ∝3 DNA obviously falls into this class of molecules, and one can therefore expect similar problems. However, the abovementioned workers overcame this difficulty by artificially inserting a small piece of foreign DNA into the molecule under-study. On forming heteroduplexes with the normal molecular form an easily observable single strand loop is observed at a fixed molecular position and serves as a reference point. Obviously the dual phage population of ∝3 is ideal for such studies, without further mmanipulation.

The molecular weights of the ∝3 Hind III restriction fragments give a total of 33,9Md for population ∝3.2 and 33,3Md for ∝3.1 (Fig. 12) as compared to 34,7Md determined electron microscopically, showing very good agreement between the two techniques.

The Hind III restriction pattern (Fig. 12 and 13) of the original ∝3 stock DNA (well 6) displays one faint but sharp band Hind C. This is taken as confirmatory evidence for the permuted nature of the ∝3 phage genome (Jackson et al., 1978 a and b).

Observation of 'permuted' bands in agarose gels, depends on various factors. A large permutation giving rise to a large distribution of relatively small fragments will render them invisible due to a dilution effect. Also, large fragments that are permuted may not appear dispersed, because agarose gels are relatively insensitive to changes in fragment size at high molecular weights (Helling et al., 1974). Such

arguments may explain the absence of 'permuted' bands in the restriction patterns of Eco R1 and Bam H1 (Fig. 10).

The molecular origin of the relative insertion or deletion is of interest. One possibility is that the insertion is unstable and due to an insertion sequence (IS element). The observed relative insertion (or deletion) in the DNA of the α 3 phage is $\sim$ 1,04 kilobases in length (2 percent of 51,98 kilobases). Known IS elements of a similar size are IS1 to IS5 (Saedler and Ghosal, 1978). If such an IS element is involved then the population containing the insertion may be unstable due to precise excision of the element (Kleckner, 1977 ; Starlinger and Saedler, 1976). A recent model has been proposed to explain the transposition process (Shapiro, 1979). This model postulates that all transposable elements replicate during transposition and therefore remain at their original insertion site whilst 'jumping' to another position. However, this model does not serve to explain the observed phenomenon of reversion to wild type of mutations caused by IS elements (Kleckner, 1977 ; Starlinger and Saedler, 1976).

Alternatively the insertion or deletion may be due to a stable recombination event. At this stage, no experiments have been conducted to determine the molecular origin of the dual phage population. Such an investigation may prove valuable in future. Perhaps repeated rounds of subculturing of the separated populations will provide information as to the stability of the insertion/deletion.

In conclusion, the circularly permuted nature of the ∝3 genome is of interest in view of the known generalised transducing ability of this phage (Woods and Thomson, 1975) as a circularly permuted genome is a feature of phages capable of generalised transduction (MacHattie and Gill, 1977 ; Susskind and Botstein, 1978). This study, therefore, serves to extend this property of a phage to yet another species of bacterium. Previous observations have been confined to phages of E.coli (Streisinger et al., 1964 and 1967), Salmonella (Susskind and Botstein, 1978) and Proteus (Pretorius and Coetzee, 1979). The fact that a dual phage population was demonstrated suggests that the phage ∝3 genome can be altered in its DNA content, while retaining viability. The present study offers no explanation for the high frequency of unstable transductants peculiar to the Vibrio system (Woods and Thomson, 1975). No h.f.t. lysates (Hayes, 1968) have been isolated from Vibrio (Woods and Thomson, 1975). These workers postulate that this may be due to inadequate screening. The present study suggests, from the molecular structure of the phage ∝3 genome, that such h.f.t. lysates should be obtainable and is therefore in agreement with the abovementioned explanation for failure to isolate such a lysate. The present work may also prove of value in elucidating phage growth on stationary phase cells if a mutant phage, incapable of such growth is isolated. Such mutation could result in alteration of the mutant ∝3 denaturation map as compared to the wild type map, presented in this dissertation.

APPENDIX

DENATURATION SOLUTION

Formaldehyde	3%
Sodium Acetate	10mM
Triethanolamine	80mM

STORE as 10% formaldehyde in above buffer and buffer (as above) at 4°C.

DROPLET SOLUTION

Formamide	30%
Sodium Acetate	0,1M
Triethanolamine	50mM
pH (8,0) with acetic acid	

STORE at 4°C.

ELECTROPHORESIS BUFFER (XI)

Tris HCl	40mM
Sodium Acetate	50mM
EDTA	10mM
ph (7,7)	

LURIA SALT BROTH (LSB)

Bacto Tryptone	10g
Bacto Yeastextract	5g
Analar NaCl	23,4g
water	1 litre

LURIA SALT AGAR (LSA)

LSB	1 litre
Oxoid Agar No. 3	12g

LURIA SOFT SALT AGAR (LSSA)

LSB	1 litre
Oxoid Agar No. 3	6g

RESTRICTION BUFFERS (XI)

Eco R1

Tris HCl	0,1M
NaCl	50mM
$MgCl_2$	10mM
pH(7,5)	

Bam H1

Tris HCl	0,1M
$MgCl_2$	10mM
pH(7,5)	

Hind III

Tris HCl	6mM
NaCl	50mM
$MgCl_2$	6mM
BSA	1mg/ml
pH(7,5)	

SALINE

NaCl	23,4g
water	1 litre

SSC (XI)

NaCl	0,15M
Na citrate	0,015M
pH(7,0)	

TE BUFFER

Tris HCl	10mM
EDTA	10mM
pH(8,0)	

Unless otherwise stated, all chemicals were from Merck.

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