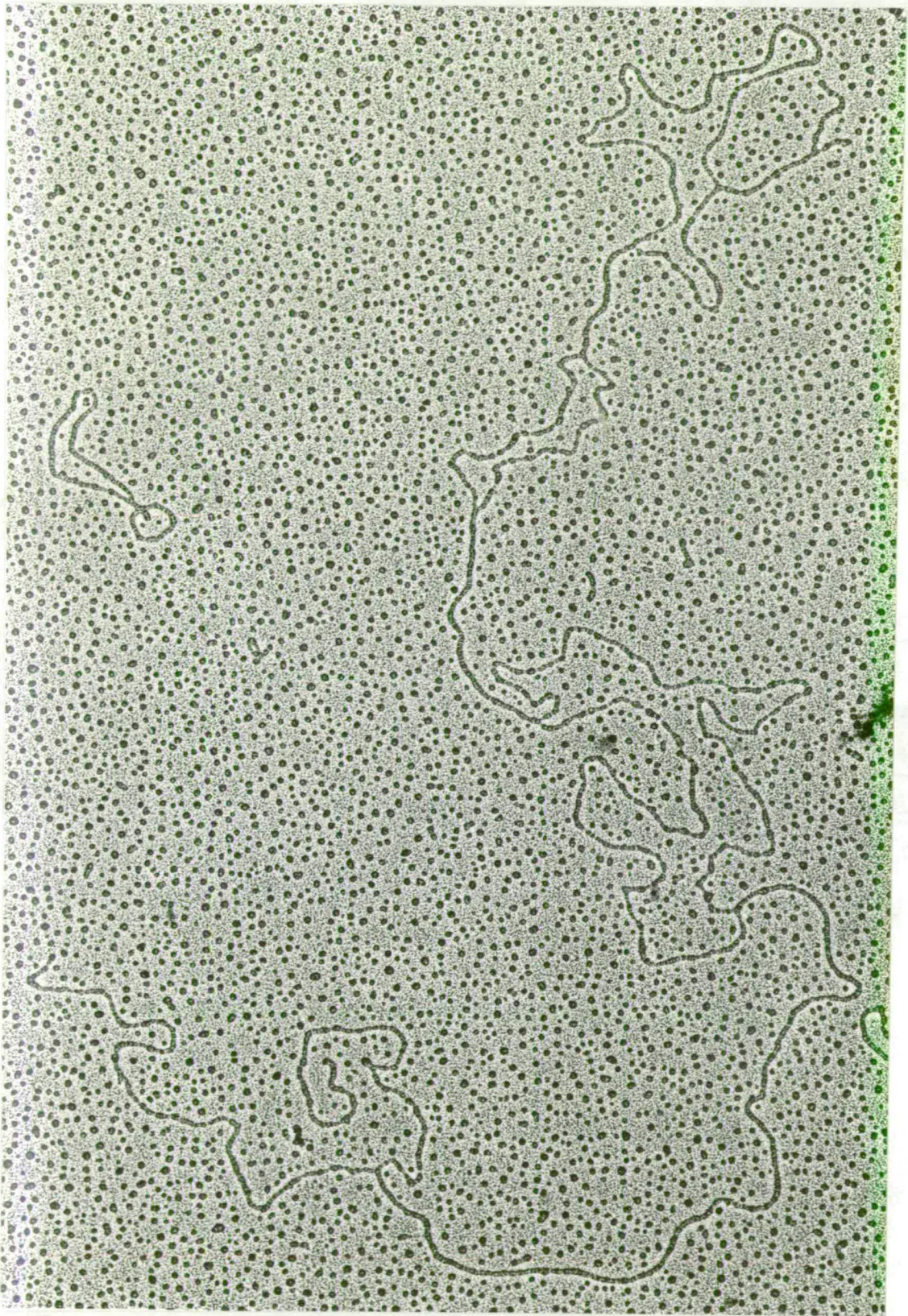# THE EVOLUTION OF LAMBDOID PHAGE GENOMES:

the Exchange of DNA Segments Between Phages

and with the _E.coli_ Chromosome

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The heteroduplex between the DNA molecules of the phages 82 and 21.

This thesis was composed by myself and the work was my own.

Yenhui Chang

CONTENTS

ABSTRACT

Twelve of the fifteen possible heteroduplexes between the DNA molecules of the six phages λ, 434, 82, PA2, 21 and 424 have been previously made and analysed by electron microscopy. The last three, 82/PA2, 82/21 and 82/424, were made in this work. Each heteroduplex showed a number of regions of homology. These homologies between the phages define 32 segments which compose a common genome. For each segment there are several allelic alternatives which are functionally homologous. These observations suggest that the phages have evolved from a common ancestor and have exchanged alternative alleles with one another. But the possibility that the different phages found in nature may have all evolved independently from a common ancestor has not been eliminated. Evidence for exchange of alleles would be four natural strains that exhibit all four possible combinations of two allelic alternatives in two separate regions of the genome. The addition of the last three heteroduplexes revealed three examples of combinations indicating exchanges among the six phages. However, the segments involved were very short and of unknown function. For further evidence, 33 new phages were isolated from clinical samples which were from patients in the Lothian Region. By analyzing the heteroduplexes made with new and old phages, twelve different types of combination of alleles indicating exchanges were found, several of which involved alleles of known function.

In the course of making heteroduplexes I found small insertions, possibly IS elements, in the genomes of phages 82, 434, and λ. The size of these insertions was in the range of 1.2 to 1.5 kilobases.

Gene lc was first found in the phage PA2. It codes for a protein, named protein 2, which appears on the outer membrane of the bacterium after the phage PA2 DNA has integrated into the cell chromosome. Mutants of E.coli K-12 strain, called nmpC(P+), produce a new outer membrane protein NmpC, which is closely related structurally and immunologically to protein 2. I generated lambda phages with substitutions from the qsr' prophage of a wild type and a mutant nmpC(P+) E.coli K-12 strain. The substitutions from the two strains were homologous except that there was an extra piece of DNA which was about 1,300 base pairs in length in the one from the the wild type strain. This insertion was in a part of the substituted DNA which was also homologous with a region in the phage PA2 genome which contains the gene lc. The phage without the insertion in the substitution enables its lysogen to produce an extra outer membrane protein which is very similar to protein NmpC and also to protein 2, which is the product of lc gene. The nmpC gene has been mapped at 12 min on the E.coli chromosome and the qsr' prophage is known to lie nearby. I conclude that in fact the nmpC gene lies within the qsr' prophage, and that in wild type strains the expression of this gene is prevented by the insertion.

SECTION 1. EVIDENCE FOR THE EXCHANGE OF SEGMENTS BETWEEN PHAGES AND WITH THE E.COLI CHROMOSOME DURING THEIR EVOLUTION. INSERTIONS IN PHAGE GENOMES.

INTRODUCTION

Since bacteriophage λ was isolated by Lederberg (1951), a number of phages resembling λ have been isolated. Among them, phages 21, 82, 424 and 434 isolated by Jacob and Wollman (1956) have been studied by Simon et al (1971) and phage φ80 isolated by Matsushiro (1963) is widely used. These phages have three properties in common: they recombine when intercrossed, their DNA molecules possess identical pairs of cohesive ends, and their prophages are inducible by ultraviolet irradiation (Hershey and Dove, 1971). The members of this group of phages are often called lambdoid phages. The electron microscopic heteroduplex technique was applied to study the structure of the DNA molecules of these phages and the relationships between them. Simon et al (1971) made heteroduplexes between the DNA molecules of λ and of 434, 82 and 21, and between those of 434 and 82. Each heteroduplex showed a number of regions of homology and in most non-homologous regions the two strands of DNA differed in length. From these data Hershey and Dove (1971) deduced three notable conclusions. First, DNA molecules of the phages contain segments that appear to be either identical with segments of λ DNA or completely different; only a few partially matching segments are found. Second, the identical sequences in a given pair of DNAs add up to 35% to 60%

of the total molecular length, depending on the pair. Third, identical segments occupy positions characteristic of the pair and lie approximately equidistant from the left molecular end in both members of the pair. Fiandt et al (1971) made the heteroduplexes between the DNA molecules of λ and φ80 and showed that recombination between the λ and φ80 molecules occurred in the homologous regions. These results led Hershey and Dove (1971) to postulate that the DNA molecules of this λ family are functionally homologous and have a common genome. They also led to the idea (Hershey, 1971b) that the several races were derived from still other races by reassortment of unlike segments, and that both the diversity and the capacity to form hybrid recombinants have served a joint evolutionary purpose. DNA segments brought in from outside the λ family by illegimate recombination (Franklin, 1971) may have played a dominant role in the family history.

The evolution of such a family of phages was discussed by Campbell (1977). He suggested that the clustering by function of genes within λ reflects the evolutionary origin of the phage genome from its component segments. He postulated two possible extreme alternative evolutionary processes. Either the phages may have evolved from a common ancestor with the same general gene arrangement as seen today, or the genomes may have been independently constructed by association of nonviral elements arranged in the same order, each of which was picked up from a number of functionally similar replicons differing in sequence. He extended the reassorting hypothesis to include not only exchanges of segments between viral varieties but also between viral chromosome and host chromosome, or between viruses and other extrachromsomal replicons. He pointed out that although

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observations suggest the occurrence of recombination, and although it is possible for recombination to occur in the laboratory, the possibility that different natural lines may have each evolved independently from a common ancestor has not been eliminated. The most convincing evidence for a recombinational origin of existing strains would be natural strains that exhibit all four possible combinations of two allelic alternatives in two separate regions of the genome (Fig 2.4b). With such an arrangement, no family tree can be drawn relating all individuals except by postulating independent recurrence of specific changes. This is tenable for any change that can happen in a few mutational steps but is very unlikely for extensive base-sequence differences recognizable in heteroduplexes.

Aiming at finding out whether exchanges of segments had really occurred between the DNA molecules during their evolution and how many alleles there might be for each segment, Myers (1980) set out to make heteroduplexes of all possible combinations of λ , 424, 434, 21, 82 and PA2. She concluded that there was a common genome among the lambdoid phages which was composed of discrete segments. The number of alleles of most segments is small. An evolutionary scheme was deduced to explain the existing relationships . between the six phages in terms of direct evolution from a common ancestor, apart from one DNA exchange.

This example of exchange involves a segment (30), one end of which is not defined by a boundary between homologous and non-homologous regions in two of the phages involved (434 and PA2). The segment could be missing in these two. So, this is not rigid evidence for DNA

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exchange between lambdoid phages.

Heteroduplexes of phage 82 with phages 424, PA2 and 21 were not done in Myer's work. I set out to make these heteroduplexes first and to look for further evidence of exchange.

MATERIALS AND METHODS

Table 1.1.   Bacterial Strains

Strain	Relevant features	Source	Reference
C600	K-12	N.Murray and C.Ma	Appleyard(1954)
W3110	K-12		Yanofsky and Ito (1966)
NM259	K-12, supIII (suppressor of $\underline{Sam}$7 mutation in λ).	K.Murray	
C600(82)	lysogenic for 82	N.Murray	
C600(424)	lysogenic for 424	N.Murray	
C600(21)	lysogenic for 21	N.Murray	
W1485($\lambda\underline{cI}$ 857$\underline{Sam}$7)	W1485, lysogenic for $\lambda\underline{cI}$857$\underline{Sam}$7.	K.Murray	

Table 1.2.   Bacteriophage Strains

Strain	Relevant features	Source	Reference
$\lambda\underline{b}2\underline{imm}^{21}\underline{p}4$	$\underline{p}$4 insertion	N.Murray	Fiandt $\underline{et}$ $\underline{al}$ (1971)
PA2$\underline{ts}$9	clear plaque temperture sensitive mutant of PA2	R.Myers	Schnaitman $\underline{et}$ $\underline{al}$ (1975)
82 clear	clear plaque mutant of phage 82	N.Murray	Arber(1960)
$\lambda\underline{b}$1	$\underline{b}$1 insertion	E.R.Singer	Kellenberger $\underline{et}$ $\underline{al}$ (1960)

Bacterial and Bacteriophage Strains (Table 1.1 and Table 1.2)

Phages λ, 434, 82, 21 and 424 were induced by UV and phage PA2 by heat. Phage stocks were prepared from liquid lysates and then precipitated with PEG 6000.

Media

The following were sterilized by autoclaving at 15 1b/in^2 for 15 minutes before use.

L broth	Difco Bacto tryptone	10g
(pH 7.2)	Difco Bacto yeast extract	5g
	NaCl	5g
	Distilled water	to 1 litre
L agar	Difco Bacto tryptone	10g
(pH 7.2)	Difco Bacto yeast extract	5g
	NaCl	10g
	Difco agar	15g
	Distilled water	to 1 litre
BBL agar	Baltimore Biological Laboratories	
	(BBL) trypticase	10g
	NaCl	5g
	Difco agar	10g

	Distilled water	to 1 litre
BBL top layer	BBL trypticase	10g
	NaCl	5g
	Difco agar	6.5g
Phage buffer	K_2PO_4	3g
	Na_2HPO_4 (anhydrous)	7g
	NaCl	5g
	$MgSO_4$ (0.1M solution)	10ml
	$CaCl_2$ (0.01M solution)	10ml
	1% gelatin solution	1ml
	Distilled water	to 1 litre

UV Induction of Bacteriophages

Overnight cultures of lysogenic bacteria in L broth were diluted 20x in L broth, regrown for about 2h and harvested in a benchtop centrifuge. The cells were then resuspended in half the original volume of 0.01M $MgSO_4$ and exposed to UV radiation for a dose of 400ergs/mm^2. After being diluted four-fold into fresh warm L broth, the cells were allowed to grow at 37°C in the dark with good aeration for two hours. Chloroform was added to the culture (2 drops per 5ml) to lyse surviving cells. After 5 min, cell debris was removed by centrifugation (4,000 rpm for 10 min) in a benchtop centrifuge. The supernatant was titrated.

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Heat Induction of CS419 (a Thermosensitive Lysogen)

A thermosensitive lysogen carries a prophage containing a temperature sensitive mutation in cI gene. At the restrictive temperature (usually about $42^{\circ}C$), the product of this gene, namely the repressor, will be defective. So the prophage will be induced. A fresh overnight culture of CS419, a PA2 thermosensitive lysogen of E.coli W1485, was diluted 50-fold into L broth and incubated at $32^{\circ}C$ until the absorbance at 650 nm in a 1 cm cell was 0.45. The flask was then transferred to a shaking water bath at $42^{\circ}C$ to inactivate the repressor. After 20 to 30 min , the flask was transferred to $37^{\circ}C$ with continual shaking in order to maintain good aeration for 2 h. Chloroform was added to the culture (2 drops per 5 ml) to lyse any remaining intact cells. The culture was shaken for 5 min and the debris was spun down at 4,000 rpm for 10 min in a benchtop centrifuge. The supernatant was titrated.

Preparation of Phage Stocks by Liquid Lysate

Overnight cultures of cells in L broth were diluted 20x in L broth and regrown for about two and half hours. The cells were then concentrated 10-fold into the same medium by centrifugation. Phage at a m.o.i. of 0.05 were added (assuming the cell concentration to be 5×10^{9}/ml) and allowed to adsorb for 15 min at room temperature. The cells were diluted 60-fold into L broth containing 0.1% glucose and 2 mM $MgSO_4$. Lysis occurred in about 1.5 to 2 h. After adding $CHCl_3$ and shaking for 5 min, the lysate was clarified by centrifugation at 4,000 rpm for 10 min. The supernatant was titrated.

Concentration of Phage by Polyethylene Glycol (PEG) Precipitation

To a clarified phage lysate prepared as above, DNAase was added to give a final concentration of 1 µg/ml. This was left to stand at 37°C for 2 h. Then solid NaCl and PEG 6000 were added to give final concentrations of 0.7 M and 10% w/v respectively and dissolved by stirring. The solution was stored at 4°C for at least 1 h (it may be left overnight). Phage were spun down at 4000 rpm for 15 min. The pellet was resuspended in phage buffer. (Phage lysates may be concentrated up to 40 to 50-fold.) I found that the phage DNA at this stage can be extracted by adding phenol directly to the phage without any further purification.

Preparation of Phage DNA

Freshly distilled phenol was pre-equilibrated with an equal volume of 0.5 M Tris (pH 8.0). The phases were allowed to separate and the Tris layer (top) was removed, except for a thin layer to prevent air getting in. The phenol was stored at -20°C. DNA was extracted with an equal volume of phenol by mixing gently, allowing both phases to separate and finally removing the upper ˄ layer with a Pasteur pipette. aqueous? The extraction was repeated three times. Any contaminating phenol was then dialysed out against 0.01 M Tris, 0.001 M EDTA (pH 8.0) during about 20 h (1 ml DNA against 500 ml dialysis solution). Dialysis tubing was boiled for 20 min in 0.2 mM EDTA before use. The DNA was stored in acid-washed tubes at 4°C.

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DNA Heteroduplex Formation

DNA heteroduplexes were formed by denaturing two phage DNAs in alkaline solution, and then neutralizing the solution allowing the DNAs to renature. Denaturation was achieved by allowing a solution containing 3×10^{10} DNA molecules (1.5 µg) of each of the two phages in 0.5 ml of 0.1 M NaOH, 20 mM EDTA to stand for 10 minutes at 27°C. Following denaturation, 50 µl of 1.8 M Tris.HCl, 0.2 M Tris.OH was added to neutralize the solution and 0.5 ml of formamide (98.5%) was also added. The pH was 8.5 at this point. After two hours renaturation at 27°C, about 50% of the molecules were renatured. In the presence of formamide, random base interactions in single-strand DNA (i.e. intrachain hydrogen-bonding) was prevented during renaturation. The DNA was ready to spread at this stage. To store the heteroduplexes, the DNA solution was dialysed against a solution containing 10 mM Tris, 1 mM EDTA (pH 8.5) overnight. The DNA was then stored at 4°C.

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DNA Spreading for Electron Microscopy

The procedure for spreading the DNA heteroduplexes was to dilute the DNA into a freshly prepared hyperphase solution and, after the addition of cytochrome c, to spread it on to the hypophase solution which was also freshly prepared. The hyperphase and hypophase solutions were approximately isodenaturing.

The hyperphase contained 0.15 µg DNA/ml, 0.18 mg cytochrome c/ml, 0.1 M Tris, 0.01 M EDTA and 50% formamide (pH 8.5). When standards with known lengths were necessary, circular double-standed plasmid

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pAT153 and single-stranded phage M13 DNA were added to give 0.5 to 1 μg/ml final concentration of each standard. Cytochrome c was used to form the protein film on the surface of the hypophase which binds the DNA. The structure visualized in the electron microscope is a column of protein around the DNA. This structure is about 100 A thick. The hypophase contained 0.01 M Tris, 0.001 M EDTA, 15% formamide (pH 8.5). Since it may become acidic relatively quickly, the hypophase solution was made up shortly (5 minutes or less) before use.

A clean silica ramp was put into a teflon trough containing the hypophase solution. Fifty microlitres of the hyperphase solution was slowly released close to the ramp-solution boundary from a 50 μl Eppendorf pipette. The solution ran down the ramp on to the hypophase and formed a surface film with DNA bound to it. The film was allowed to stand for 1 minute before being picked up to achieve better spreading of single-stranded DNA. The film was picked up near the ramp-solution boundary with electron microscope grids coated with parlodion film, stained in 0.002% uranyl acetate in 90% ethanol and rotary shadowed with platinum. The specimens were then coated with carbon, to give stability on irradiation in the electron microscope, and the parlodion was removed by immersion in 100% ethanol for 60 seconds.

Electron Microscopy and Measurement

Specimens were viewed with a Siemens Elmiskop 1a and photographed at x10,000 to x20,000 magnification, with an accelerating voltage of 80,000 volts. For each type of heteroduplex, twenty photographs were taken to minimize the errors due to distortion during spreading and drying. At least three of each type of standard adjacent to each heteroduplex were photographed. Negatives were projected with a photographic enlarger at a further x5 magnification and measured directly with a Ferranti Cetec tablet digitiser linked to an Olivetti P6040 calculator. The measurements of heteroduplexes were calculated relative to the measurements of standards and were then expressed in $\%$ λ units.

The sum of the double and single-stranded regions of the two molecules in a heteroduplex was generally slightly different from their known lengths in $\%$ λ units. The variation in the lengths of the single-stranded regions was greater than that of the double-stranded regions and it was assumed that being more flexible the single-stranded regions were most subject to distortion. So correction factors were applied to the single-stranded regions to give the correct total lengths.

RESULTS

Heteroduplexes of 82 with PA2, 424 and 21

The structure of these three heteroduplexes are shown in Figure 1.1. For each heteroduplex, twenty molecules were measured to minimize errors. The length of each region was averaged. For single-stranded regions, the longer strands of each bubble were averaged together and the shorter strands of each were averaged together. The lengths of the 82 and 21 molecules were measured by Simon et al (1971) and the length of the 424 molecule was determined by Highton and Whitfield (1975). The assignment of single strands in the bubbles was based on predictions made from other heteroduplexes and agreement of the total lengths (Appendex 1.2). From any pair of heteroduplexes,which have one molecule in common, it is possible to predict some of the regions of homology and non-homology in a heteroduplex between the other two molecules. Two examples are given here to show how it was done. In the heteroduplex λ/82, the two molecules are nonhomologous in the immunity region, and λ is 5.1% longer than 82 in this region. In the heteroduplex λ/21, λ is 5.0% longer than 21 in the immunity region. Thus, one can predict that in the immunity region of heteroduplex 82/21, 21 is 0.1% longer than 82. So the longer strand of this bubble was assigned to the 21 molecule. In the heteroduplex 424/82, there is a very big bubble. The length of 82 DNA molecule is 91.5% λ and that of 424 is 97% λ. Only one way, that is to assign the shorter strand to 82, can make 82 shorter than 424. The positions of the boundaries in the heteroduplexes are given in Appendix 1.1.
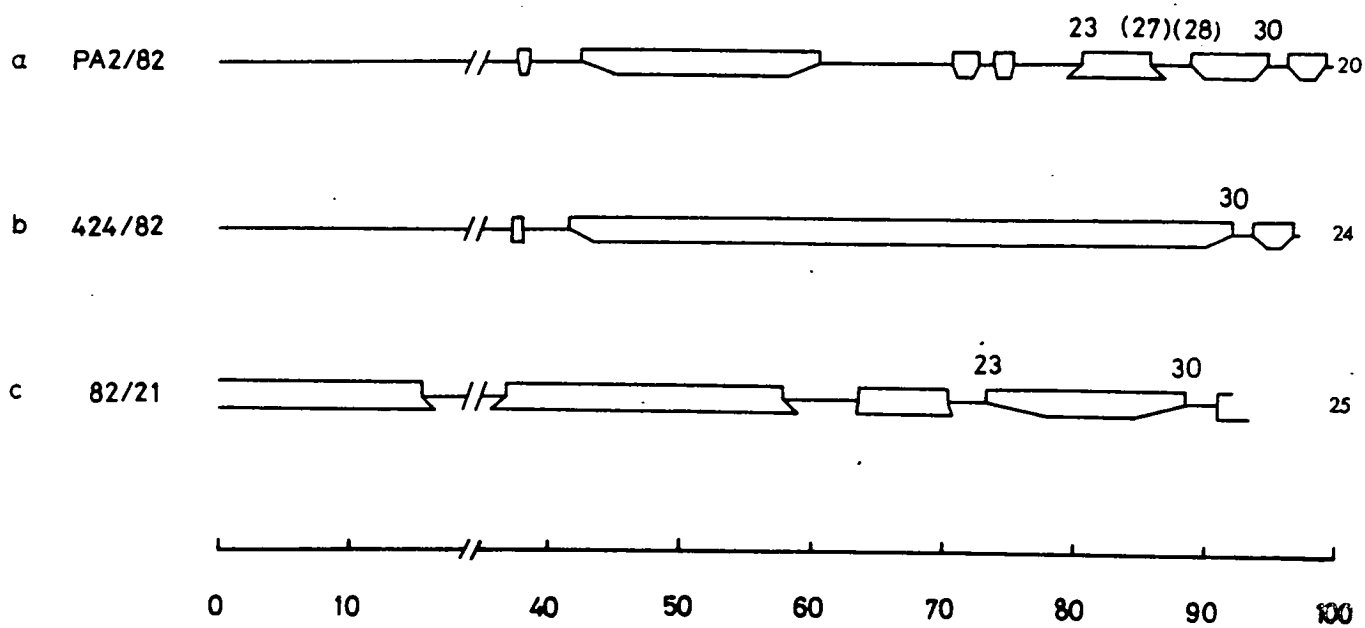
Figure 1.1 Heteroduplexes with the phage 82 DNA molecule. (The number of molecules measured is given on the right; boundary positions are in Appendix 1.1; mode of assignment of single strands is in Appendix 1.2.)

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The Common Structure of the Six Phage Genomes

All the heteroduplexes that can be made between the DNA molcules of the six phages λ, 434, 82, PA2, 424 and 21 are shown in Figure 1.2. Four of them were made by Simon et al (1971), three were made in this work; seven were made by Myers (1980) and one by Highton (unpublished). The regions of homology in the different heteroduplexes are related to one another by vertical lines. These are drawn connecting the boundaries between homologous and non-homologous regions which are in corresponding positions. For example, in both the λ/82 and λ/PA2 heteroduplexes there are boundaries at 68.9% in the λ molecule. The position of these boundaries in the 82 and PA2 molecules agree closely with the position of boundaries in the heteroduplexes between them and the 434 and 21 molecules. In these heteroduplexes the positions of the boundaries in the 434 molecule agree closely with one another, as do those in the 21 molecule. These six boundaries are all called number 15. The positions of the boundaries in the heteroduplexes are given in appendix 1.1.

Some lines joining corresponding boundaries are not completely vertical because of the limitation of accuracy of the method used. The justification for joining boundaries which differ in position is based on the predictable homology relationships. For example, boundaries 1 and 2 in the 434/PA2, 434/424, PA2/82 and 424/82 heteroduplexes, although slightly displaced relative to one another, must all correspond because the PA2 and 424 molecules are homologous in this region and so are the 434 and 82 molecules. Similarly, boundaries 3 and 4 in the λ/434, λ/82, λ/PA2 and λ/424 heteroduplexes must correspond, because
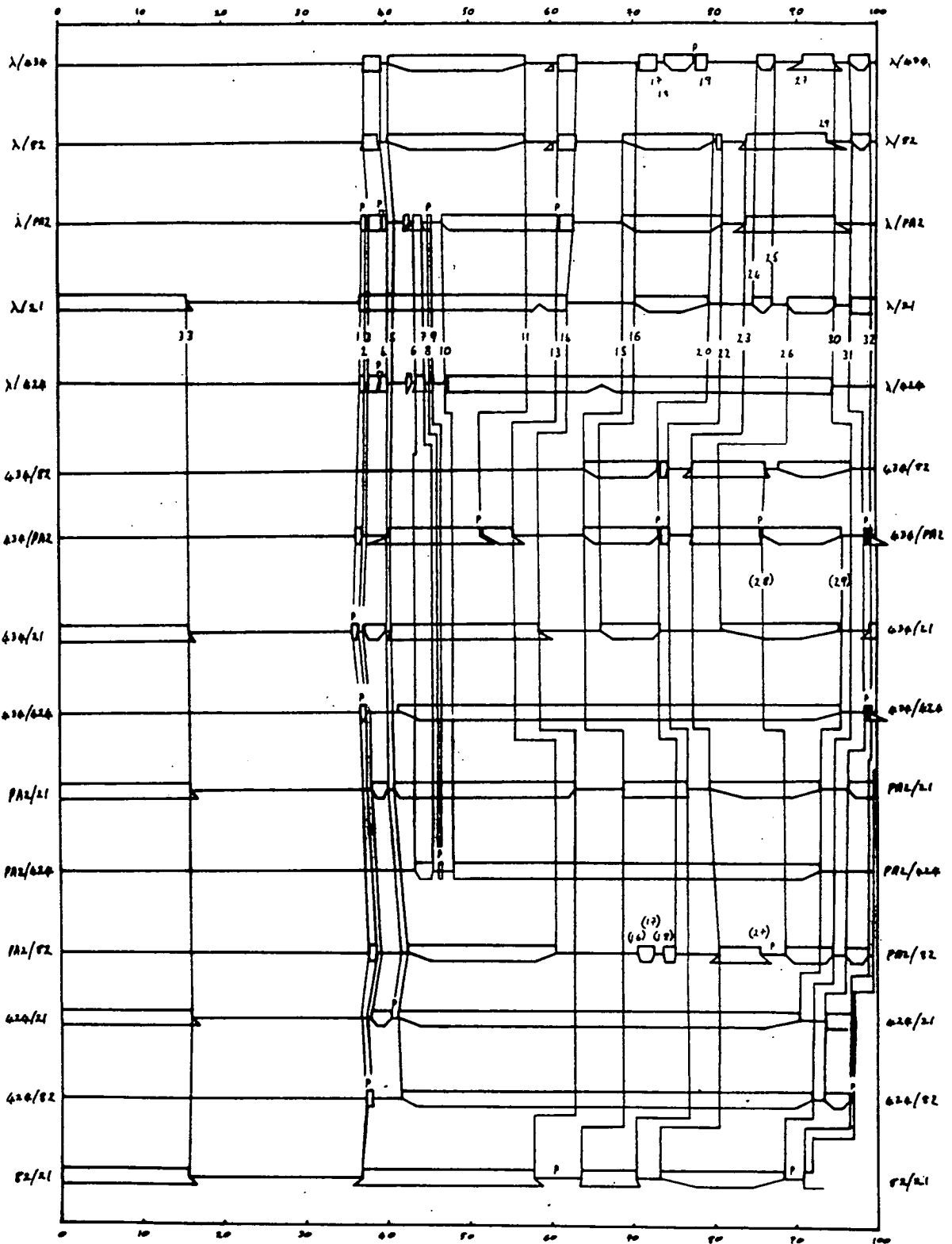
Figure 1.2 The fifteen possible heteroduplexes between the DNA molecules of phages λ, 21, 82, 424, 434 and PA2. [λ/434, λ'/82, λ/21, 434/82 (Simon et al, 1971); λ/424 (Highton, unpublished); λ/PA2, 434/PA2, 434/21, 434/424, PA2/21, PA2/424, 424/21 (Myers, 1980); PA2/82, 424/82, 82/21 (This work)]. The vertical lines connect corresponding boundaries.

434, 82, PA2 and 424 molecules are all homologous with each other in this region.

The homologies between the six phage DNA molecules reveal a common structure. The lines joining corresponding boundaries define a common genome composed of segments for most of which there are two or more allelic alternatives. This is represented in Table 1.3. Since the heteroduplex analysis of the six phages does not define all the boundaries in every phage, the division of each molecule into the same number segments is based on the assumption that they have functionally homologous genomes. That does not necessarily mean that all the molecules have the same number of segments. In each molecule a few segments could be absent. For example, segments 6 to 10 are clearly present in the λ, PA2 and 424 molecules (Figure 1.2), but in the heteroduplexes between these three and the others only one relevant boundary, number 5, is present. Thus any of these segments could be missing in the 434, 82 or 21 molecules. In Table 1.3 the boundaries in each genome that are defined by the heteroduplexes are shown by vertical lines. In several places a boundary is defined in two or more phages but cannot be related to the boundary in λ. These are shown by broken lines. The numbers of these boundaries are shown in brackets in Figure 1.2.

Table 1.3. The occurrence of the alternative alleles

for each segment of the commom genome

Phage   Segment number (same as right boundary[c] number)

Phage	33b	1	2	3	4	5	6	7	8	9	10	11	13	14	15	16
λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ
434	λ	λ	λ	λ	434	λ	434	434	434	434	434	434	λ[b]	434	λ	λ
82	λ	λ	λ	λ	434	λ	434	434	434	434	434	434	λ[b]	434	λ	82
PA2	λ	λ	PA2	λ	434	λ	λ[a]	PA2	λ	PA2	λ	PA2	PA2	434	λ	82
21	21	λ	PA2	λ	21	λ	21	21	21	21	21	21	21	21	λ	λ
424	λ	λ	PA2	λ	434	λ	λ[a]	424	λ	424	λ	424	424	424	424	424

Phage	17	18	19	20	22	23	24	25	26	27	28	29	30	31	32	33a
λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ
434	434	λ	434	λ	λ	λ	λ	434	λ	λ	434	434	434	λ	434	λ
82	82	82	82	82	82	λ	82	82	82	82	82	82	λ	λ	434	λ
PA2	PA2	82	PA2	PA2	82	λ	PA2	PA2	PA2	PA2	82	PA2	434	λ	λ	λ
21	21	21	21	21	λ	λ	λ	434	λ	21	21	21	434	λ	434	21
424	424	424	424	424	424	424	424	424	424	424	424	424	434	λ	λ	λ

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a. 0.6% deleted relative to λ.

b. 0.9% inserted relative to λ.

c.   Solid lines define boundaries which can be related to positions in
the λ molecule.   Broken lines define boundaries which can be related to
one another but not to positions in the λ molecule.

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Combinations of Alleles Indicating Exchanges

The evidence of exchanges between phage genomes given by Myers (1980) involves segment 30. In the genomes of 434 and PA2, we can not relate boundary 29 to that in λ and 82 (Table 1.3). Thus the region of homology to the left of the boundary 30 in the heteroduplex 434/PA2 (Figure 1.2) is not necessarily one of the allelic alternatives of segment 30 in λ and 82. Segment 30 could be missing in 434 and PA2. We can only be sure that a segment is present in a molecule if both boundaries are defined in it by the heteroduplexes.

After the completion of all the fifteen heteroduplexes between these six phages, three examples of exchange were found. They are shown in Table 1.4. Both boundaries of each segment involved in this table are defined by heteroduplexes.

Two exchanges depend on the new heteroduplexes, and one which involves 2 and 32 was not seen by Myers (1980). That was because segment 32 in phage 21 was mistaken as 21 type. In fact this segment is homologous with 434, i.e., 434 type. This is confirmed by the 82/21 heteroduplexes which shows that 82 is homologous with 434 in this segment.

Table 1.4. Combinations of alleles (boxed)

indicating exchanges between genomes

Phage	Segment number		
	2	22	32
			(6, 8, 10)

Phage	2	22	32
λ	λ	λ	λ
434	λ	λ	434
82	λ	82	434
PA2	PA2	82	λ
21	PA2	λ	434

The Correlation Between Segments and Genes

Figure 1.3 shows the correlation between the segments and the genetic map of λ (Sanger et al, 1982). Most boundaries lie between the genes quite precisely. But boundaries 1, 2 and 3 appear to lie clearly within gene J, and 22 within gene O. All the head genes are in segment 33b and all the tail genes except part of gene J are in segment 1. The integration and excision genes and their site of action (int, xis, attP) are all in segment 13, and the recombination genes exo, β, γ and gene kil are in segment 15. Genes cIII, ssb and ral are in segment 16, gene N is in segment 17. The rex genes, the operators O_L and O_R and the repressor genes cI and cro are in segment 19, and gene cII is in segment 20. Genes P and ren are in segment 23 and gene Q and S are in segment 29. Gene R is in segment 30, and gene Rz is in segment 31.

In fact from the comparison of the sequences of λ and λi434 (Yocum et al, unpublished), boundary 18 is known to lie further to the left than determined by Simon et al (1971, see Figure 1.3) and so part of gene N may lie in segment 18. The other end of the immunity region, boundary 19, lies between cro and cII (Grosschedl and Schwartz, 1979) as determined by Simon et al (1971).

Some genetic evidence can be given to support the correlation between the segments and the genetic map. It is known that phages λ, 434 and 82 integrate at the same site in the E.coli chromosome, i.e. 17 min. Phage PA2 integrates at 50 min (Pugsley et al, 1979), 21 at 27 min and 424 at 44 min (Jacob and Wollman, 1961). Also Gottesman and

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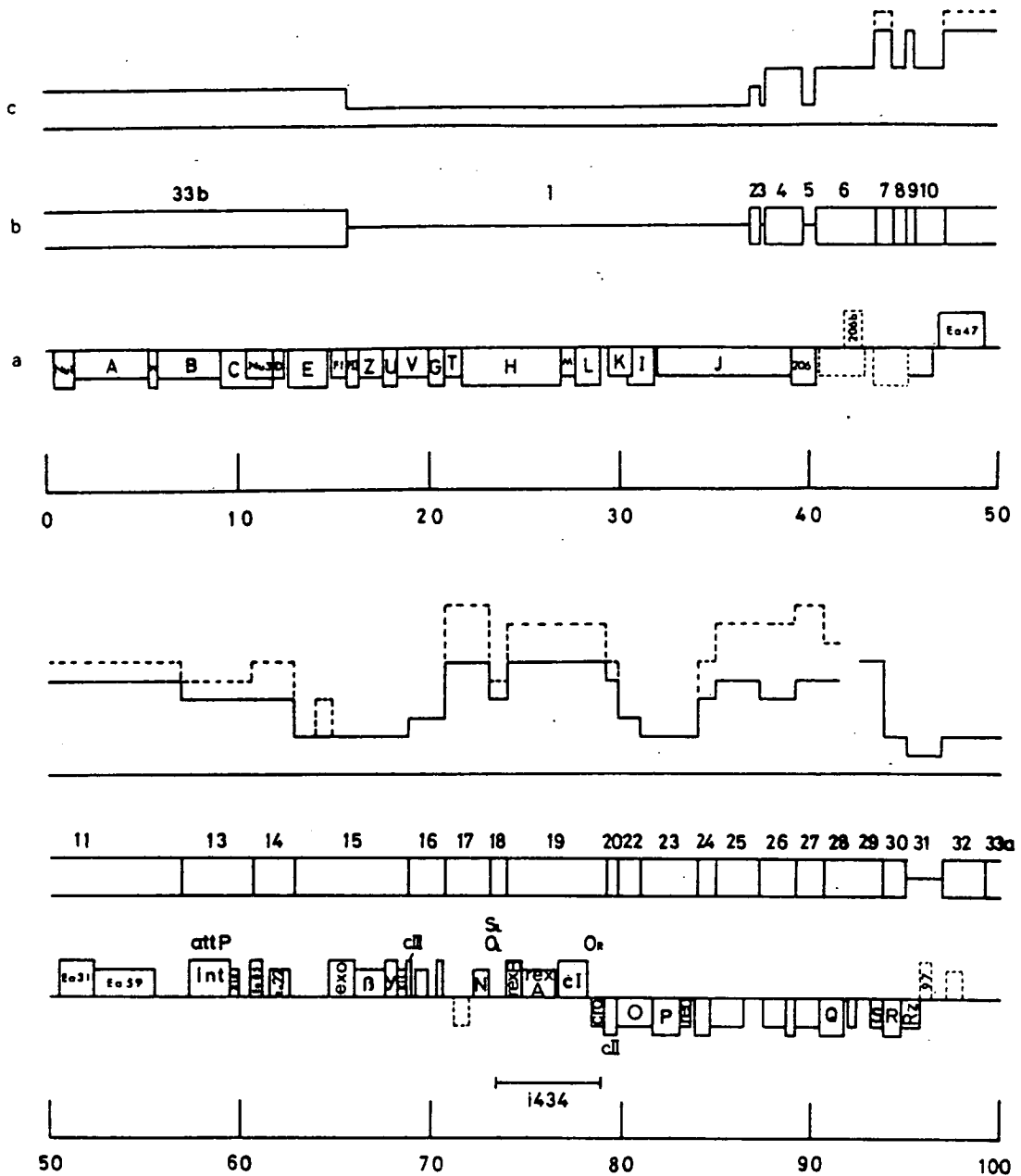
Figure 1.3 The correlation between segments and genes.

a.   The genetic map of phage λ (Sanger,1982).

b.   The segmentation of the λ genome.   Boundary positions are deduced from the five λ heteroduplexes in Figure 1.2.   (Segments are numbered according to their right boundary.)

c.The allelic diversity of each segment deduced from the six phage genomes included in Figure 1.2 (see Table 1.3) and six newly isolated phages (see Table 2.6).   The dashed line indicates further possible alleles (see Table 2.6 footnote a).

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Yarmolinsky (1968) have shown that 434 can complement lysogens for λ defective in _int_ function but not 21 lysogens. Correspondingly, in segment 13 where _attP_, _int_ and _xis_ lie λ, 434 and 82 are homologous, PA2, 424 and 21 are non-homologous with the other three (~~Figure~~ ᵀᵃᵇˡᵉ 1.3).

Kaiser and Jacob (1957) reported that turbid plaque recombinants carrying the immunity of λ could be obtained from crosses of _cIII_ mutants of λ with 434 and 21, but not 82. Gene _cIII_ is just to the right of boundary 15. We can look at the relationship between the molecules to check whether the allocation of _cIII_ into segment 16 is correct. In segment 15 which accommodates the recombination genes the λ, 434 and 82 molecules are homologous. This is supported by the observation that the λ exonuclease and β protein are immunologically the same in λ,434 and 82 (Radding and Schreffler, 1966; Radding _et al_, 1967). So _cIII_ can only be in segment 16.

Boundary 19 and 20 are very close together, but they are not the same one as the gene _cII_ lies beween them. From the heteroduplexes in Figure 1.2, we can deduce that the _cII_ gene in λ is the same as that in 434 but different from that in the others. This is supported by Kaiser and Jacob's report (1957) that turbid plaque recombinants with the immunity of λ are obtained from crosses of _cII_ mutants of λ with 434, but not with 21.

Dove (1969) reported that 434 can complement λ in the DNA replication functions, _O_ and _P_, and 82 can complement _P_ but not _O_ of λ. This is consistant with the fact that part of gene _O_ is in segment 22 and the remainder together with gene _P_ is in segment 23. In

segment 22, λ is homologous with 434 but not with 82, and in segment 23 λ is homologous with 434 and 82. The origin of replication lies in the middle of O, so conceivably this divides the protein into two functional halves which have alternative alleles.

In addition to the genes of known function there are five of unknown function. Of these Ea47, Ea31 and Ea59 lie in segment 11, and Ea85 and Ea22 lie in segment 14. Finally, a number of open reading frames for which no protein product is known have been identified from the sequence (Sanger et al, 1982). Again these mostly lie within segments. The exceptions are in the region spanning segments 4 to 10. However, a more satisfactory agreement is obtained if these segments are moved slightly to the left. This also brings boundary 4 into better agreement with the end of gene J. Region 4 is known to determine host specificity which is also known to be determined by this end of the J protein (see section 3). Given the variation that exists in the positions determined for these regions in the different heteroduplexes (see Figure 1.2) this correlation is quite possible.

Recently the sequence of the immunity region of phage 434 (segment 19) has been compared with that of λ. The left end is at the start of the major leftward transcript (S_L) (Yocum et al, unpublished) and the right end is 9 bases beyond the end of cro (Grosschedl and Schwartz, 1979).

Insertions in the Phage Genomes

Insertions, approximately 2.7% λ units in length, were detected in the course of making heteroduplexes. They apparently occurred spontanously during subculturing in the laboratory. These insertions were detected in the DNA molecule of phages λ, 434 and 82, three out of five lambdoid phages which had been studied by the heteroduplex technique.

a) The Insertion in the 82 Genome

This was detected in the 82/PA2 heteroduplex (Figure 1.1). In a non-homologous loop [segment 24 to (27)], the 82 DNA molecule was longer than predicted from the λ/PA2 heteroduplex (Myers, 1980) and the λ/82 heteroduplex (Simon et al, 1971).This extra length also appeared in the 82/424 and 82/21 heteroduplex (Figure 1.1). The difference between the 82 used by Simon et al (1971) and mine (called 82') was confirmed by making the λ/82' heteroduplex. The increased length was in the region in 82 corresponding to 84 to 94% in λ. This region of λ has twice been found to exchange with a larger region in the E.coli chromosome called the p4 substitution (Jacob. and Wollman, 1954; Herskowitz and Singer, 1970; Sato and Campbell, 1970). So, I thought that the same change might have occurred with 82. The phage had been propagated as a lysogen for many years. However, the λp4/82' heteroduplex showed that the 82' region was homologous with the p4 substitution over only part of its length, and there was a small loop about equal to the increased length in 82' towards the left end of this region (Figure 1.4a). Subsequently, I obtained another stock of
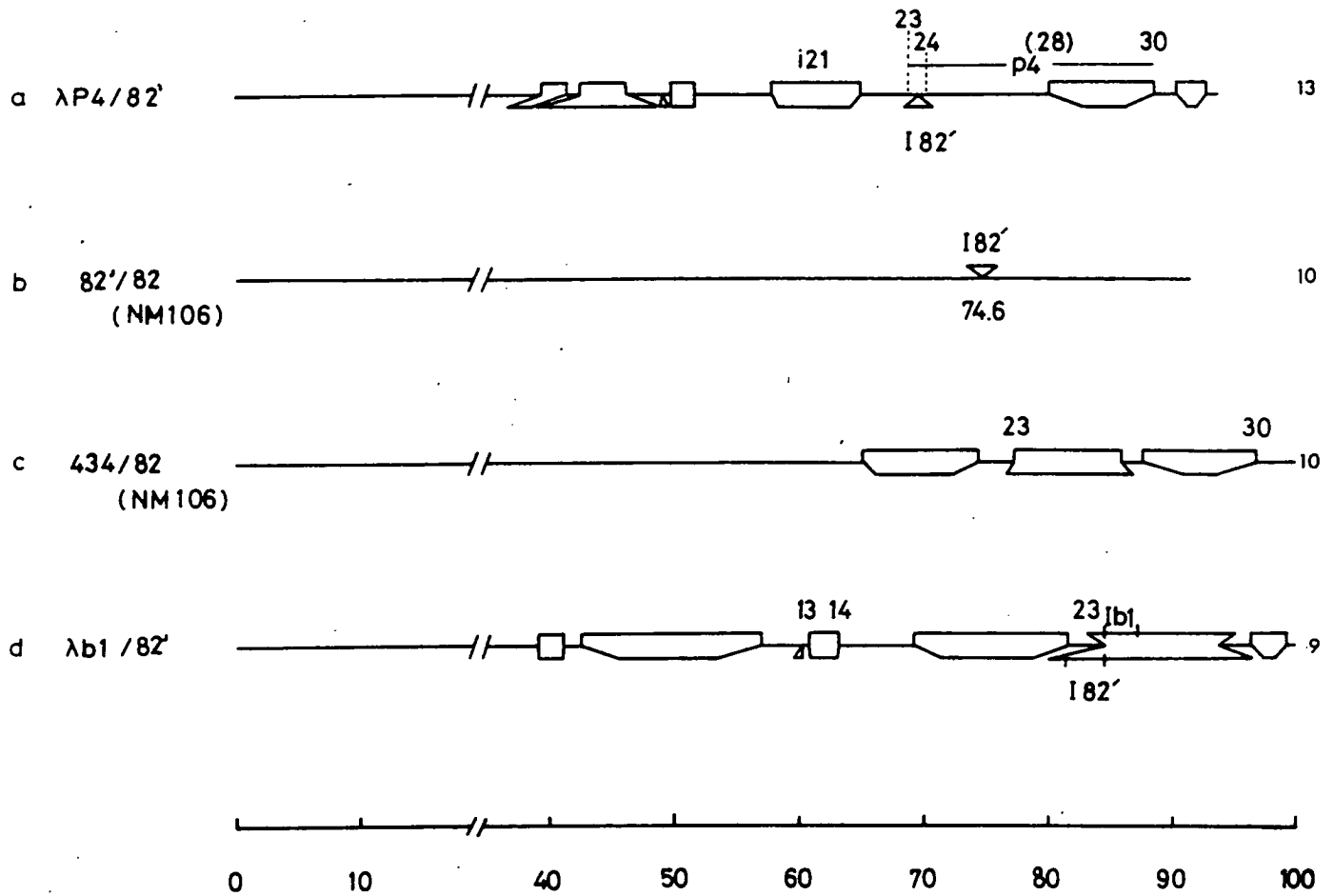
Figure 1.4 Heteroduplexes showing the insertion I82ʹ in the phage 82ʹ DNA molecule and the homology of the phage 82ʹ DNA molecule with the p4 region of the E.coli chromosome. (The number of molecules measured is given on the right; boundary positions are in Appendix 1.1; mode of assignment of single strands is in Appendix 1.2.)

phage 82 called NM106. This had been propagated as free phage for many years. The heteroduplex 82'/82(NM106) showed a small loop 2.7% λ units in size at the position of 74.6% (Figure 1.4b). In the heteroduplex $\lambda\!\!\diagup\!\!\overset{p4}{\underset{\lambda}{}}$/82(NM106), the homology with the $\underline{p}$4 substitution was as with 82', but the small loop was absent. From the 434/82(NM106) heteroduplex (Figure 1.4), 82(NM106) appears to be the same as Simon's 82 (Figure 1.2). So, it was clear that there was an insertion (I82') in the 82' DNA molecule and that the 82 molecule was homologous with part of the $\underline{p}$4 substitution. (The insertion loop was also seen in a heteroduplex between 82' and a stock of 82 obtained from E. Wollman.)

The size and position of this insertion was very similar to the $\underline{b}$1 insertion in λ from E.$\underline{coli}$ K12-112, which has been shown to be an IS2 (Zissler $\underline{et}$ $\underline{al}$, 1977). I thought that the 82' insertion might also be an IS2. The heteroduplex $\lambda\underline{b}$1/82' was made but showed no homology in the region where I expected to see homology (Figure 1.4d). Further work needs to be done to characterize the 82' insertion.

b) The Insertion in the λ Genome

In the course of the above work, I made the heteroduplex λ/$\lambda\underline{p}$4 (Figure 1.5a). This was made with λ DNA from a W1485 ($\lambda\underline{cI}$857$\underline{S}$7) lysogen. An unexpected small loop was found at 61.9%. When this λ DNA was used to make the heteroduplex λ/82', an extra length was shown in a loop just past 60% (Figure 1.5b). This extra length did not exist in the heteroduplex $\lambda\underline{p}$4/82'(Figure 1.5c) or $\lambda\underline{b}$1/82'(Figure 1.4d). These results suggested that this λ (called λ') had an insertion (Iλ') in

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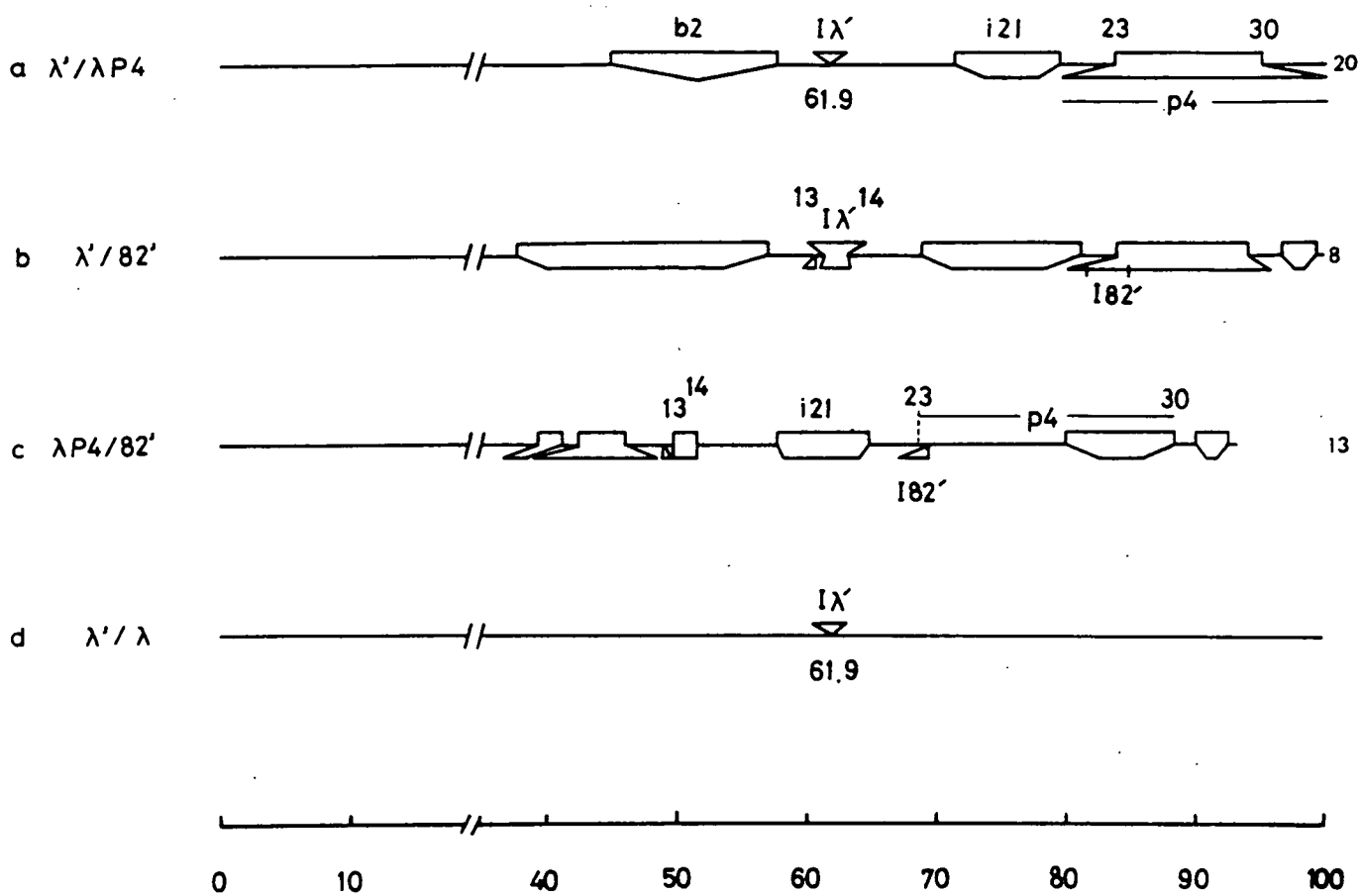
Figure 1.5 Heteroduplexes showing the insertion Iλ' in the phage λ' DNA
molecule. (The number of molecules measured is given on the right;
boundary positions are in Appendix 1.1; mode of assignment of single
strands is in Appendix 1.2.)

its genome, which was measured as 2.7% λ in length. The insertion was
not in DNA previously obtained from the stock from which the λ'
lysogen had descended or in DNA obtained earlier from a lop8 lysogen
[conclusions from λ'/λ (Figure 1.5d) and λ(lop8)/82' heteroduplexes].

An insertion of similar position and size called bi2, 2.8% λ in length,
at position of 61.6%, was detected by Mosharrafa et al (1976) and
shown to be an IS2. Like the one in λ', it appears to have occurred
spontaneously during subculturing. It was detected by a change in the
gel pattern of an EcoRI digest, and was not present in the λcI857S7
from four other laboratories.

c) The Insertion in the 434 Genome

This was detected as an extra loop in the heteroduplex between
the DNA of phage from two plaque types. After UV induction of the
C600(434) lysogen, two types of plaques were found on strains W3110
and 259. One was large and clear, the other was small, faint and
turbid. The ratio of the former to the latter was approximately 1 to
10. However, these two types were not distinguishable when grown on
C600. To see whether a structural difference could be detected, a
heteroduplex was made between the DNA of phage propagated from a
single clear plaque (on W3110) and that from a UV induced culture
(Figure 1.6b). Although an insertion was found in the heteroduplex
preparation, it occurred with low frequency and thus seemed unrelated
to plaque morphology. By making heteroduplexes between the DNA of
phage 82 and each of these two stocks of 434 DNA, it was shown that
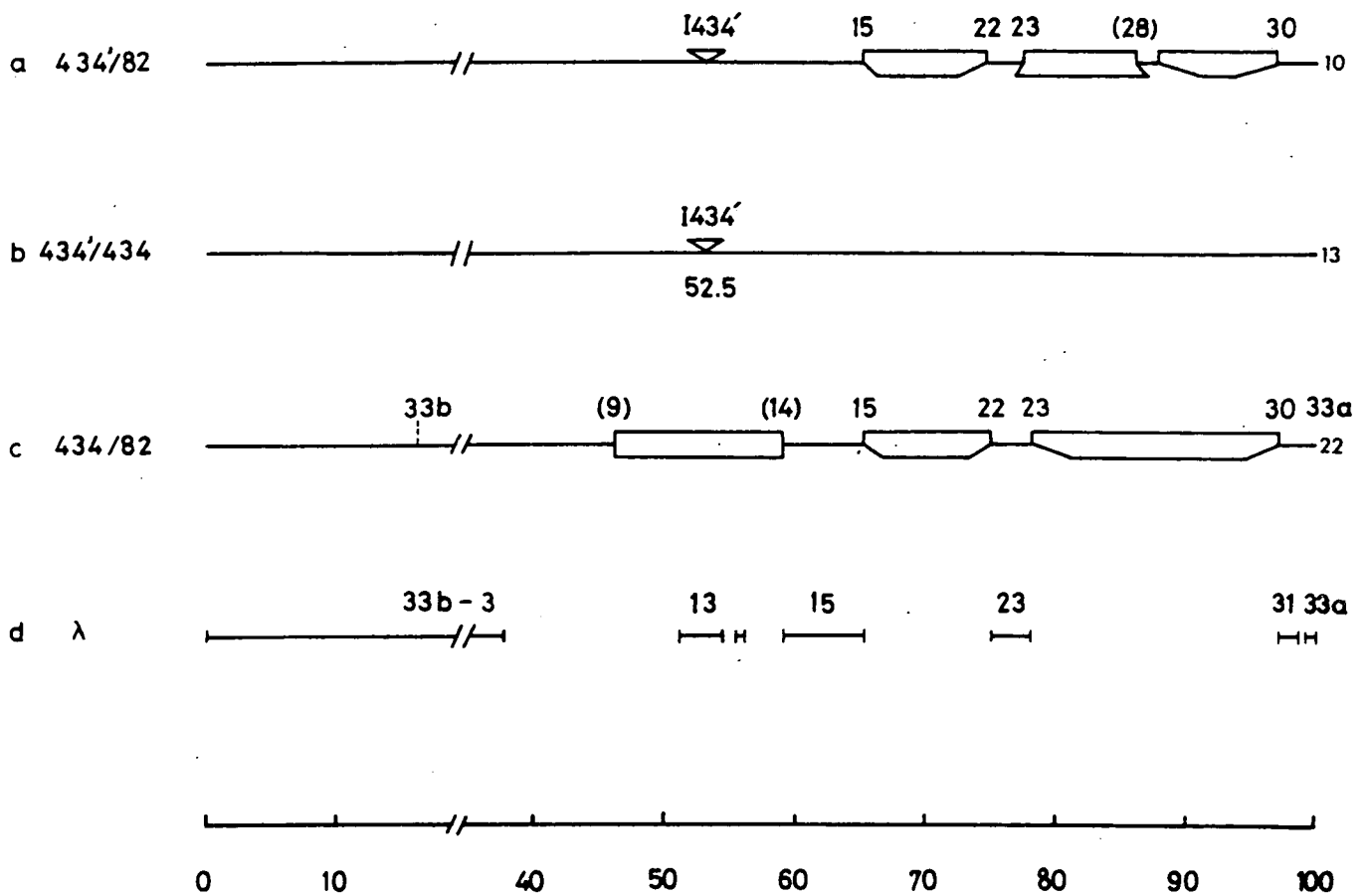
Figure 1.6 Heteroduplexes showing the I434' insertion in the 434' DNA molecule, and the stability of the 434/82 heteroduplex. a) and b) hyperphase - 50% formamide, c) hyperphase - 80% formamide, d) regions of the λ DNA molecule homologous with the 434 and 82 DNA molecules (see Figure 1.2). The number of molecules measured is given on the right.

there was no insertion in the DNA from the clear plaque and that 1 to 5% of the molecules from the UV induced culture had the insertion (called I434') (Figure 1.6a). This was confirmed by denaturing and self-annealing this DNA.

The insertion was about 2.6% λ in length, at a position of 53%, which corresponds to 59% in λ, where the int gene is located. It may also be an IS2 insertion.

Mismatch in the Homologous and Non-homologous Regions of Heteroduplexes

The heteroduplexes between lambdoid phages are observed to be composed of stretches of homology and stretches of non-homology, and only a few short regions are partially homologous under the standard spreading conditions. From an evolutionary point of view, it is necessary to find out just how much mismatch there may be in both "homologous" regions and non-homologous regions.

a) Mismatch Deduced from Spreading in Different Denaturation Conditions

The heteroduplex 434/82 which has a 64% long region of homology starting at the left end was chosen to be analyzed for mismatch in "homologous" regions by spreading it in different formamide concentrations. The spreading solution (hyperphase) contained 0.1 M Tris, 0.01 M EDTA, which gives a cation concentration of 0.06 M. The cation concentration is calculated from the ionization constants of Tris and EDTA at the pH of 8.5 (Davis and Hyman, 1971). The hypophase

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contained 0.01 M Tris, 0.001 M EDTA, which gives a cation concentration of 0.006 M. The concentration of formamide in the spreading solutions were 60%, 70%, 80% and 85%, and in the hypophase, they were 37%, 47%, 57% and 62% respectively, so that isodenaturation could be attained. That is, the denaturing conditions of the spreading solution are identical to those of hypophase. The calculation of formamide concentrations was based on the equation:

$$Tm = 176 - (2.60 - X_o)(3.60 - 7.04\log_{10} [Na^+])$$ (Frank-Kamenetskii, 1971).

Tm is the melting temperature of the DNA and X_o is the fractional GC content of the DNA. From this equation, it was worked out that:

Tm for a 10 fold cation concentration decrease = Tm for a 23% formamide increase.

So the formamide concentrations in the hypophase were 23% lower than those in the spreading solutions. The spreadings were carried out at 28°C, and phage 434 DNA was also spread with 85% and 90% formamide in the spreading solution as a control of homoduplex denaturation.

The results showed that segments 10 to 14, 15, 23, 29, 31 to 33a in the 434/82 heteroduplex were denatured in at least some molecules in 80% formamide (Figure 1.6c); in the homoduplex, only segments 10 to 14 were denatured in 80% formamide. No segments were denatured in 70% formamide. From the relationships that 1% formamide lowers the Tm by 0.65°C, and 1% mismatch lowers the Tm by 1.4° C (Hyman et al, 1973), an equation can be worked out:

1% formamide = 0.46% mismatch.

With this equation, it can be worked out that the mismatch in the "homologous" regions of the heteroduplex 434/82 is less than 4.6% (Table 1.5). The segments to the left of number 10 were not denatured in

Table 1.5

Segment	Percent formamide required for denaturation		Percent mismatch deduced from columns 2 and 3	Segment	GC content(%) deduced from homology with the λ genome	Percent mismatch deduced from the GC content
	Homoduplexes	Heteroduplexes				
33b-9	>90%	85%(variable within region)	>2.3	33b-9	56.2	1.25
10-14	80%(100)[a]	80%(100)	0	13	43.2	-0.57
15	90%(100)	80%(18)	<4.6	15	47.9	1.06
23	90%(100)	80%(41)	<4.6	23	50.5	1.70
29	85%(100)	80%(100)	2.3	29		
31-33a	85%(100)	80%(23)	<2.3	31	44.5	-0.24
				33a	41.2	-1.21

a) The numbers in brackets are the % of the molecule in the sample which were denatured in this region.

90% formamide in the homoduplex but some parts were denatured in 85% formamide in the heteroduplex. So the mismatch in these segments is more than 2.3%.

There is also a way which allow us to deduce the percentage of mismatch in those homologous regions in 434/82 which are homologous with the λ genome. In the 434 and 82 genomes, the segments 33b to 9 (partly), 10 to 14 (partly), 15, 23, 31 and 33a are homologous with λ. The GC contents of these regions in λ calculated from the λ sequence (Sanger et al, 1982) are 56.2, 43.2, 47.9, 50.5, 44.5 and 41.2 respectively. The DNA molecules of 434 and 82 are assumed to have the same GC content as λ in these regions. The Tm of these regions can be calculated from the GC content according to the equation:

$$Tm = 176 - (2.60 - X_o)(3.60 - 7.04\log_{10} [Na^+]).$$

The percentage of formamide required for denaturation of a region of given GC content can be worked out with the equation

$$\% \text{ formamide} = \frac{Tm - 28^{\circ}C}{0.65}$$

where $28^{\circ}C$ is the temperature used for DNA spreading. The percentage of mismatch for each region can be obtained by working out the difference between the concentration of formamide required for heteroduplex denaturation and that predicted for homoduplex denaturation, and then multiplying it by 0.46 (Table 1.5).

The result worked out by the second method is consistant with that by the first one. They both show that the percentage of mismatch in homologous regions of the heteroduplex is low. But

because the predicted formamide concentration required for homoduplex denaturation is lower than that needed for heteroduplex denaturation in the experiment, the value of mismatch in segments 10 to 14, 31 and 33a are negative. The explanation for this may be as follows,

1) The predicted Tms may be too small or the factor 0.65 is slightly large. If 0.62 is used instead of 0.65, the mismatch deduced from the two ways is brought to the same level. The values in the third column ✗ in Table 1.5 will decrease slightly, but the last column will increase like this:

Segment	Mismatch
33b-9	3.1
10-14	1.4
15	2.77
23	3.41
31	1.47
33a	0.5

2) The GC contents in 434 and 82 are higher than that in λ DNA.

3) The small errors in measurement of temperature or formamide concentration.

Another experiment in which the $\lambda/424$ heteroduplex, which has a 48% long region of non-homology, was spread at $4^{o}C$ in a spreading solution containing 30% formamide (Highton, unpublished) showed that in the single stranded regions there was at least 50% mismatch.

a) Mismatch Detected by Sequence Comparision

The sequencing of the λDNA molecule by (Sanger et al, 1980), and the imm434 region of the 434 molecule (Grosschedl and Schwartz, 1979; Yocum et al, unpublished) made it possible to see the mismatch of the λ and 434 DNA molecules in this region by sequence comparison. Grosschedl and Schwartz (1979) sequenced the 434 molecule from 512 bases to the right of the imm434 region, through to the first twelve bases of the cI gene (Figure 1.3). Yocum et al (unpublished) sequenced an overlapping 1404 base pair HpaII piece of the λimm434 DNA molecule, running from 120 bases to the left of the imm434 region, through to the first six codons of the 434 cro gene. By comparing the sequence of these regions with the λ sequence, it was found that the 120 bases at the left end of the 1404 base pair HpaII piece, are homologous with the λ sequence, from positions 35583 to 35464, except for single base substitutions at three places, and one base insertion. This region must be 434 DNA, and must be within segment 18 which is homologous to λ in the heteroduplex λ/434 (Figure 2.1). The recombination event which produced λimm434 must have occurred further to the left in segment 18. The right end of the 120 bp homologous region is also S_L, the start of leftward transcription, and must correspond to boundary 18 (see Figure 1.3).

On the right of the imm434 region, there are 512 base pairs in 434 genome which are 98% homologous with the λ genome. The corresponding λ region is from positions 38247 to 38758. It includes cII and the flanking intercistronic regions, and also the beginning of gene O. This region is segment 20.

Segment 19, which is from S_L to the far end of <u>cro</u> in the λ genome, and which is replaced by imm434 in λimm434 is non-homologous in the heteroduplex λ/434. Grosschedl and Schwartz (1979) found "no obvious homology between the λ and 434 gene sequences". However, by computer analysis of the λ and 434 <u>cI</u> gene sequences Yocum <u>et</u> <u>al</u> (unpublished) did find several short regions with greater than 50% homology, one of which may correspond to a region of partial homology found by Simon <u>et</u> <u>al</u> (1971) (Figure 1.2).

From these results, it can be concluded that in the homologous regions 18 and 20 of the λ/434 heteroduplex, the two strands are almost identical; in the non-homologous region 19, the two strands are very different.

DISCUSSION

In the six phages analyzed above, only three examples of combinations indicating exchanges were found, and the segments involved in them are very short. We can argue that two homologous alleles may simply be identical insertion elements which inserted in two phages at corresponding positions. So, more rigid evidence for exchange is needed. A strong reason for believing that exchanges may have occurred is that it is not possible to devise a very satisfactory evolutionary scheme without invoking exchanges.

The evolutionary scheme proposed by Myers (1980) was designed to explain the existing relationships as far as possible by "point mutation" from a common ancestor. This involved the "stop and starting" theory, that is, one part of the phage genome started to mutate and then stopped; another part then started and then stopped, and the former site started again. That seems unlikely. To avoid the "stopping and starting" it is necessary to invoke exchanges. If exchanges have occurred during the evolution, the homology between phages can be explained as the result of recombination. This will of course also mean that it is impossible to work out an evolutionary scheme to try to explain the existing relationships between the lambdoid phages. Because of the reasons above, I decided to isolate more phages and look for more evidence of exchanges.

Small insertions, possibly IS elements, were found in the genomes of phages 82, 434 and λ . The addition of IS elements has previously been observed at a number of points in the λ genome (Zissler et al,

1977) and may play an important role in the evolution of bacteriophages. The report about λaltSF, a variant of phage λ with an IS1 inserted in the <u>cI</u> gene, which acquired the ability to substitute new sets of genes from <u>E.coli</u> genome (Friedman <u>et al</u>, 1981) suggests that the IS element plays a role in the substitution process. This is an efficient means for rapid virus evolution. (There are some criticisms about the work of λaltSF. See Section 3, Results.)

Appendix 1.1   The Positions of the Boundaries in the Heteroduplexes in Figures 1.1, 1.4[a], 1.5[a]

Heteroduplex	33	1	2	3	4	5	6	7	8	9	10	11	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Figure 1.1																																
a. PA2/82			37.7	38.5		42.3							60.4			70.5	72.6	73.7		75.1		80.4						88.7		94.4	96.0	98.8
			37.7	38.4		42.2							54.8			64.9	66.4	67.4		68.6		74.0						84.7		88.3	89.9	90.7
b. 424/82			37.2	37.9		41.6																								91.8	93.3	96.4
			37.2	38.1		41.7																								88.4	89.9	90.9
c. 82/21	15.4	36.9												57.7	63.5							70.2	73.2					88.2			90.7	
	16.6	38.0												61.4	67.2							74.5	77.5					84.1			86.6	
Figure 1.4																																
a. λp4/82'				39.4	41.4	42.5						46.1	49.6	51.5	57.7					64.9								80.0		88.4	90.2	92.6
				39.4	41.5	42.6						52.0	56.2	58.0	64.2					70.2								84.8		88.0	89.5	90.8
c. 434/82															65.0							74.4	77.3					85.9		96.8		
															65.0							71.4	74.3					84.5		88.2		
d. λb1/82'				39.1	41.2	42.6						57.0	60.8	63.1	69.2							81.6	84.6								96.4	99.2
				39.1	41.3	42.6						50.5	55.0	57.1	63.2							70.2	73.2								90.0	90.8
Figure 1.5																																
a. λ'/ p4																71.3				74.3		83.6								95.0		
																59.0				63.0		67.3								88.2		
b. λ'/82'				37.7								57.0	61.3	63.0	68.8							81.0	83.8							93.9	96.5	99.2
				37.7								51.1	56.4	58.5	64.3							71.4	74.2							87.3	89.9	90.7
c. λp4/82' (see Figure 1.4a)																																

Header spanning "Boundary" ... "Number" over the numbered columns.

[a] The insertions in λ', λb1 and 82' are not included.

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Appendix 1.2. Assignment of strands in the non-homologous regions

in Figures 1.1, 1.4, 1.5.

Heteroduplex   Heteroduplexes usd for assignment

Figure 1.1

a. PA2/82' λ/PA2[b] λ and λ/82[a]

                                434/PA2[b] and 434/82[a]

b. 424/82' λ/424[c] λ and λ/82[a]

                                434/424[b] λ and 434/82[a]

c. 82/21' λ/82[a] λ and λ/21[a]

                                434/82[a] and 434/21[b]

Figure 1.4.

a. λp4/82' λ/82[a] and λ'/λp4[d]

c. 434/82   434/82[a]

d. λb1/82' λ/82[a]

Figure 1.5.

a. λ'/λp4 λ/21[a]

b. λ'/82' λ/82[a]

c. λp4/82'   (see Figure 1.4a)

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a Simon et al, 1971.

b Myer's,1980.

c Highton, unpublished.

d This work.

SECTION 2. THE ISOLATION OF NEW LAMBDOID PHAGES. FURTHER EVIDENCE FOR EXCHANGES BETWEEN PHAGE GENOMES DURING THEIR EVOLUTION

INTRODUCTION

In order to look for more exchanges, the isolation of new phages was necessary. An effective approach to finding exchanges would be to look for 4 phages which show the required relationships in one region, and then to look for the necessary second region by making heteroduplexes between the four. A similarity which can be readily detected is immunity. The immunity of a newly isolated phage can be compared to that of known phages by spotting on to lysogens of them. Phages with the immunity of λ, 434 and 82 have been reported (Jacob and Wollman, 1956; Parkinson, 1971; Schleuderberg, 1980). It should not be very difficult to find more in nature. (The phages isolated by Jacob and Wollman, and by Parkinson, are no longer available. Schleuderberg did not reply to my enquiry.) Ideally the second region should also have a known function as homologies could have arisen by insertion of non-functional DNA.

If the desired regions were found, the next step would be to spread the heteroduplexes showing homology in these regions in high formamide to test how complete the homology is.

I set out to isolate new phages from clinical samples and selected them by testing the immunities. Several phages were found with previously known immunities and evidence for exchanges was found.

MATERIALS AND METHODS

Bacterial and bacteriophage strains (Table 2.1 and Table 2.2)

Isolation of phage stocks

The source of phages was clinical samples, from patients in the Lothian Region, which had been streaked on to MacConkey plates. This was done to select for lactose fermenting E.coli-like organisms. Plates bearing colonial growth were obtained from the Central Microbiological Laboratory, Western General Hospital, Edinburgh.

Of several procedures followed (see Results section) for isolating phages the most successful was the following. Three ml of phage buffer was added to each plate to wash the colonies off. Fifty μl of suspension was taken from each sample and diluted 80-fold in L broth in a Wasserman tube. After standing at 37°C overnight the sample was centrifuged at 4,000 rpm for 15 min in a benchtop centrifuge. The pellet was discarded. The growth period was an attempt to obtain phage by spontaneous release from lysogens, and to amplify any free phage from the plates. The supernatant was treated with chloroform by vigorous agitation of 4 ml of supernatant and 0.2 ml of chloroform. This was then refrigerated for 30 min. One tenth ml of the upper aqueous layer was plated with 0.2 ml of TGL70 plating cells, and 2.5 ml of BBL top agar on to BBL agar plates. TGL70 is a derivative of C600 which is restriction negative (R^-) and modification positive (M^+). Thus DNA from phage grown in cells with other modification systems will not

Table 2.1.   Bacterial Strains

Strain	Relevant features	Source	Reference
TGL70	R^-M^+ (same as NM259, Table 1.1)	J. Scaife	
C600	K-12	C. Ma	Appleyard (1954)
C600(λ)	lysogenic for λ	N. Murray	
C600(434)	lysogenic for 434	N. Murray	Baldwin et al (1966)
C600(424)	lysogenic for 424	N. Murray	
C600(82)	lysogenic for 82	N. Murray E. Wollman	
C600(21)	lysogenic for 21	N. Murray E. Wollman	
CS419	EM3003, lysogenic for PA2ts9	C. Schnaitman	Pugsley et al (1979)
CS137	W1485F⁻, lysogenic for PA2	C. Schnaitman	
C600(PA2)	lysogenic for PA2	This work	

Table 2.2.   Bacteriophage Strains

Strain	Relevant features	Source	Reference
λ		N. Murray	Hershey (1971a)
λclear	clear plaque mutant of phage λ	N. Murray	Jacob and Wollman (1954)
λ<u>imm</u>^{434}	<u>imm</u>^{434}	N. Murray	Kaiser and Jacob (1957)
82clear	clear plaque mutant of phage 82	N. Murray	Arber (1960)

----

be restricted in TGL70 cells, and new phage DNA produced in TGL70 cells will be modified so that it will not be restricted on subsequent growth in C600.

Plates were incubated at 37°C overnight. The plates showing plaques were selected for further isolation. After two or more successive single plaque isolations, as many samples had more than one type of plaque, an isolated plaque was cut from the plate with a sterile Pasteur pipette and resuspended in 1 ml of phage buffer containing one drop of chloroform. One tenth ml of the upper layer was plated with 0.2 ml of plating cells to amplify the number of plaque forming units (PFU). The plaques were harvested by adding 3 ml of L broth and scraping off the top agar layer. After chloroform treatment and clarification, phage stocks which had 10^7 to 10^9 PFU/ml were obtained.

Construction of lysogens

Phages were spotted on to L plates seeded with E.coli K-12 strain C600. After incubation overnight cells from the centre of the spot were grown up in L broth and then streaked out on L plates spread with 10^9 phages to kill non-lysogens. After 24h incubation individual colonies were grown up and tested by spotting appropriate phages on them.

Testing for immunity

The λ, 434, 82, PA2, 424 and 21 lysogens of C600 were used for testing the immunities of the newly isolated phages. All the possible heteroduplexes between these six phage DNA molecules had been made already so that the number of heteroduplexes which would have to be made would be as small as possible.

Ten μl of each newly isolated phage was spotted on to the six lysogens with an Eppendorf pipette. The phages which did not grow on one or more lysogens were selected to make lysogens of C600. Then a reverse test was applied by spotting the six phages on them. The new phages which showed the same immunity as one of the six phages were further analysed by forming heteroduplexes and spreading for electron microscopy.

RESULTS

Isolation of new phages

From 121 plates of four batches of clinical samples, 33 phages were isolated. In the first batch, there were 44 plates. Phages were isolated twice by different methods. First isolation was done by growing a single colony from each plate overnight in L broth and then plating the supernatant of the culture on TGL70 to look for phages. With this method, only four phages were isolated. The second isolation was done by washing off the colonies with 3 ml of phage buffer and plating out the supernatant on TGL70. Six more phages were found with the second method.

Out of 39 plates of the second batch, ten phages were isolated by the method of washing off the colonies and plating on TGL70.

For the isolation of the third batch of samples, the method was modified further. After the colonies in the plate had been washed off, the suspension was diluted in L broth and grown up before the supernatant was plated out on TGL70 (see Materials and Methods section for details). From 17 plates, 9 phages were isolated. Two of them were isolated from the same sample. Surprisingly, when a test was done by spotting the supernatants on to TGL70, and on to C600 lysogenic for each of the six test phages, every sample was detected as harbouring phage. Some samples showed two types of phages and some phages only grew on certain lysogens.

From 21 plates of the fourth batch, four phages were isolated. Only two of them grew on C600. The method used was the same as the one used for the third batch. The low level of phage detection may be explained by the fact that the cultures were several days old.

Immunity testing

The newly isolated phages, except for seven that were stored at low concentration and lost viability, were spotted on the test lysogens. Some of them did not grow on two or more test lysogens (see Table 2.3a). When the same test was applied to the six test phages, it was also found that 434 did not grow on C600(434) or C600(424) although 424 grew on C600(434). This suggested that there was some unknown factor which inhibited the phage growth other than having the same immunity, and also that if a new phage did not grow on one or more test lysogens then possibly only one of these test phages would not grow on a lysogen of the new phage. Also lack of growth on only one test phage might not indicate homoimmunity. So, if any new phage did not grow on one or more test lysogens, a reverse test was always applied. This was to spot the phages of these lysogens on to lysogens of the new phage (Table 2.3b). Phage 97 can be given as an example. It did not grow on C600(21) and C600(PA2), but when phages 424 and PA2 were spotted on C600(97), only 424 did not grow.

With the method above, phages 15, 22 and 28 were detected to have the immunity of phage PA2; 84B to have the immunity of 82; 92 and 93 to have the immunity of 434. These were all confirmed by electron microscopy of the heteroduplexes (Figures 2.2j, 2.3g, 2.3f,

Table 2.3a. Immunity tests of new phages against standard phage lysogens

New phage	Standard phage lysogen						
	C600	C600(λ)	C600(434)	C600(424)	C600(82)	C600(21)	C600(PA2) or W1485(PA2)
0	+	+	+	-	+	+	+
9	+	+	+	+	+	+	+
14	+	+	+	+	+	+	n.d.
15 ($\underline{imm}^{PA2}$)[b]	+	+	+	+	+	+	-
18	-	+	-	-	-	-	n.d.
22 ($\underline{imm}^{PA2}$)[b]	+	+	+	+	+	+	-
28 ($\underline{imm}^{PA2}$)[b]	+	n.d.	+	+	+	+	-
40	-	+	-	-	-	-	n.d.
54	+	+	+	+	+	+	+
57	+	+	+	+	+	+	n.d.
60	+	+	+	+	+	+	-
69	+	+	+	+	+	+	+
71	+	+	+	+	+	+	+
73	+	+	+	+	+	+	+
80	+	+	?	?	-	-	+
83	+	+	+	+	+	+	+
84A	+	+	+	+	+	+	n.d.
84B ($\underline{imm}^{82}$)[b]	+	+	+	+	-	+	+[a]
92 ($\underline{imm}^{434}$)[b]	+	+	-	+	+	+	+[a]
93 ($\underline{imm}^{434}$)[b]	+	+	-	+	+	+	+[a]
94	+	+	+	+	+	+	+[a]
95	+	+	+	+	+	+	-[a]
97 ($\underline{imm}^{424}$)	+	+	+	-	+	+	-[a]
98	+	+	+	+	+	+	+[a]
99	+	+	+	+	+	-	-[a]
104	+	+	+	-	+	+	+
117	+	+	+	+	+	+	+

Table 2.3b. Immunity tests of standard phages against new phage lysogens

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Standard phage

New phage lysogen	λ	$\lambda\underline{imm}^{434}$	424	82	21	PA2
C600(0)	+	+	+	+	-	+
C600(9)	+	+	+	+	+	+
C600(22) ($\underline{imm}^{PA2}$)b	+	+	-	+	+	-
C600(54)	+	+	+	-	-	+
C600(84A)	-	+	-	?	+	+
C600(84B) ($\underline{imm}^{82}$)b	+	+	+	-	+	+
C600(92) ($\underline{imm}^{434}$)b	n.d.	-	n.d.	n.d.	n.d.	n.d.
C600(93) ($\underline{imm}^{434}$)b	+	-	+	+	+	+
C600(95)	n.d.	n.d.	n.d.	n.d.	n.d.	+
C600(97) ($\underline{imm}^{424}$)	n.d.	n.d.	-	n.d.	n.d.	+
C600(99)	n.d.	n.d.	n.d.	n.d.	-	n.d.

---

a W1485(PA2)

b These immunities have been confirmed by heteroduplex homology revealed by electron microscopy.

2.2e, 2.2g). A test of spotting each new phage on cells lysogenic for the others was also done (Table 2.4).

Phage grew better on some test lysogens than others which raised the question: what was the cause of the difference, the prophages or the hosts? (the origin of the C600 test lysogens was unknown.) A test was done by spotting the six test phages on their lysogens made from a single C600 colony. The result was consistant with that of spotting these phages on the original test lysogens. It can be concluded that it was the prophages which caused the lysogens to vary in their response to infection.

UV induction test and homology with λ

All the newly isolated phages were temperate phages for they formed lysogens. A UV induction test was applied to these phages. Half of them were shown to be UV inducible (Table 2.5). A prophage was classified as UV inducible if the irradiated culture showed a titre at least x5 that of the control culture. This assessment of inducibility is based on that used by Dhillon et al (1980) and differs from that originally used by Weigle and Delbruck (1951) which measured the percentage of induced bacteria. Most lysogens were picked and grown directly from growth within areas of lysis produced by spotting phage on to strain C600. At the time it was not appreciated that the lysogens may have been contaminated with non-lysogens sensitive to the phage. This may have resulted in a high concentration of phage in the culture before induction and an underestimate of the inducibility. However, the phage 15, 22, 28, 84B, 92, 93, 95 and 97 lysogens were

Table 2.4   Immunity tests of new phages against oneanother

Lysogen of C600

Phage	0	9	15	22	28	54	84A	84B	92	93	95	97
0	-	+	+	+	+	-	+	+	+	+	+	+
9	n.d.	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15 ($\underline{imm}^{PA2}$)[a]	-	+	-	-	-	-	-	-	+	+	+	+
22 ($\underline{imm}^{PA2}$)[a]	-	+	-	-	-	-	+	-	+	+	+	+
28 ($\underline{imm}^{PA2}$)[a]	-	+	-	-	-	-	+	-	+	+	+	+
54	-	+	+	+	+	-	+	+	+	+	+	+
84A	+	+	+	+	+	+	-	+	+	+	+	+
84B ($\underline{imm}^{82}$)[a]	+	+	+	+	+	+	+	-	+	+	+	+
92 ($\underline{imm}^{434}$)[a]	+	+	+	+	+	+	+	+	-	-	+	+
93 ($\underline{imm}^{434}$)[a]	+	+	+	+	+	+	+	+	-	-	+	+
95	+	+	-	+	+	+	-	-	-	-	-	+
97 ($\underline{imm}^{424}$)	+	+	-	+	+	-	-	-	-	+	+	-

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a   These immunities have been confirmed by heteroduplex homology revealed

by electron microscopy.

Table 2.5   Inducibility of new and old phages

Lysogen	Titre in control culture	Titre in UV induced culture	Increase
9	1.2×10^7	1×10^{10}	800x
14	1×10^7	1×10^6	0.1x
15($\underline{imm}^{PA2}$)[a] (S)[b]	3×10^5	1.5×10^7	50x
22($\underline{imm}^{PA2}$)[a] (S)	$<1 \times 10^5$, 3×10^4	2×10^7, 5×10^6	<200x, 170x
28($\underline{imm}^{PA2}$)[a] (S)	$<1 \times 10^5$, 2×10^5	2×10^5, 2×10^5	<2x, 1x
40	1×10^8	1×10^9	10x
54	1×10^8	5×10^8	5x
57	4×10^3	4×10^3	1x
60	3×10^8	3×10^9	10x
69	2×10^5	$<1 \times 10^5$	<0.5
71	1×10^7	1.5×10^7	1.5x
73	1.4×10^8, 3.5×10^6	2.8×10^8, 3.5×10^6	2x, 1x
80	1×10^7	1×10^8	10x
83	1×10^7	1×10^7	1x
84A	1×10^7	5×10^9	500x
84B($\underline{imm}^{82}$)[a] (S)	1×10^5, 2×10^6	3×10^6, 4.5×10^7	30x, 23x
92($\underline{imm}^{434}$)[a] (S)	2×10^7, 5×10^7	4×10^9, 1×10^{10}	200x, 200
93($\underline{imm}^{434}$)[a] (S)	2×10^7	4×10^9	200x
94	3×10^5	1×10^6	3.3x
95 (S)	2×10^5	2×10^8	1000x
97($\underline{imm}^{424}$) (S)	1.5×10^7	4×10^9	270x
98	6×10^9	4×10^9	0.7x
99	1×10^9	8×10^9	8x
104	2×10^7	2×10^7	1x
117	1.2×10^6	8×10^5	0.7x
λ (S)	4×10^6	1.2×10^{10}	3000x
82 (S)	3×10^6, 7×10^6	7×10^9, 2×10^9	2000x, 300x
434 (S)	5×10^5	5×10^7	100x
PA2 (S)	1×10^7, 1×10^6	1×10^8, 1×10^7	10x, 10x

a These immunities have been confirmed by heteroduplex homology revealed
   by electron microscopy.

b (S) means grown from a single colony.

grown from purified single colonies. All the phages which were UV inducible (except phage 54) were used to make DNA heteroduplexes with λ. All of them showed some homology with λ (Figure 2.1, 2.2, 2.3).

Evidence for exchanges

By studying the heteroduplexes between the new phages and λ, it was found that the genome of phages 22 and 28 were the same although the UV induction rates were different (see Table 2.5), and 92 differed from 93 only by an insertion loop which is about 2.7% λ in length, relative to 93 (Figure 2.2e). So phages 15 and 28 which had the same immunity as PA2, phage 84B, which had the same immunity as 82, and phage 92, which had the same immunity as 434, were selected for further heteroduplex study. Phage 9 was also selected because unlike all phages except 21 the left end of its genome, segment 33b, was non-homologous with that of λ (Figure 2.2b). By making the 9/21 heteroduplex the two phages were shown in fact to be homologous in this segment (Figure 2.2a). Thus there were several sets of four phages in which two alleles of this segment occurred twice. Phage 97 was not selected because less than 40% of its length was homologous with the λ genome although it had the same immunity as 424.

Heteroduplexes made with new and old phages are shown in Figures 2.2 and 2.3. (The positions of the boundaries and the method of assigning the single strands are given in Appendcies 2.2 and 2.3.) Phage 84A was involved because of the heteroduplexes 84A/84B which was done to check the homology between these two phages which were isolated from the same sample. By analysing these heteroduplexes it was found
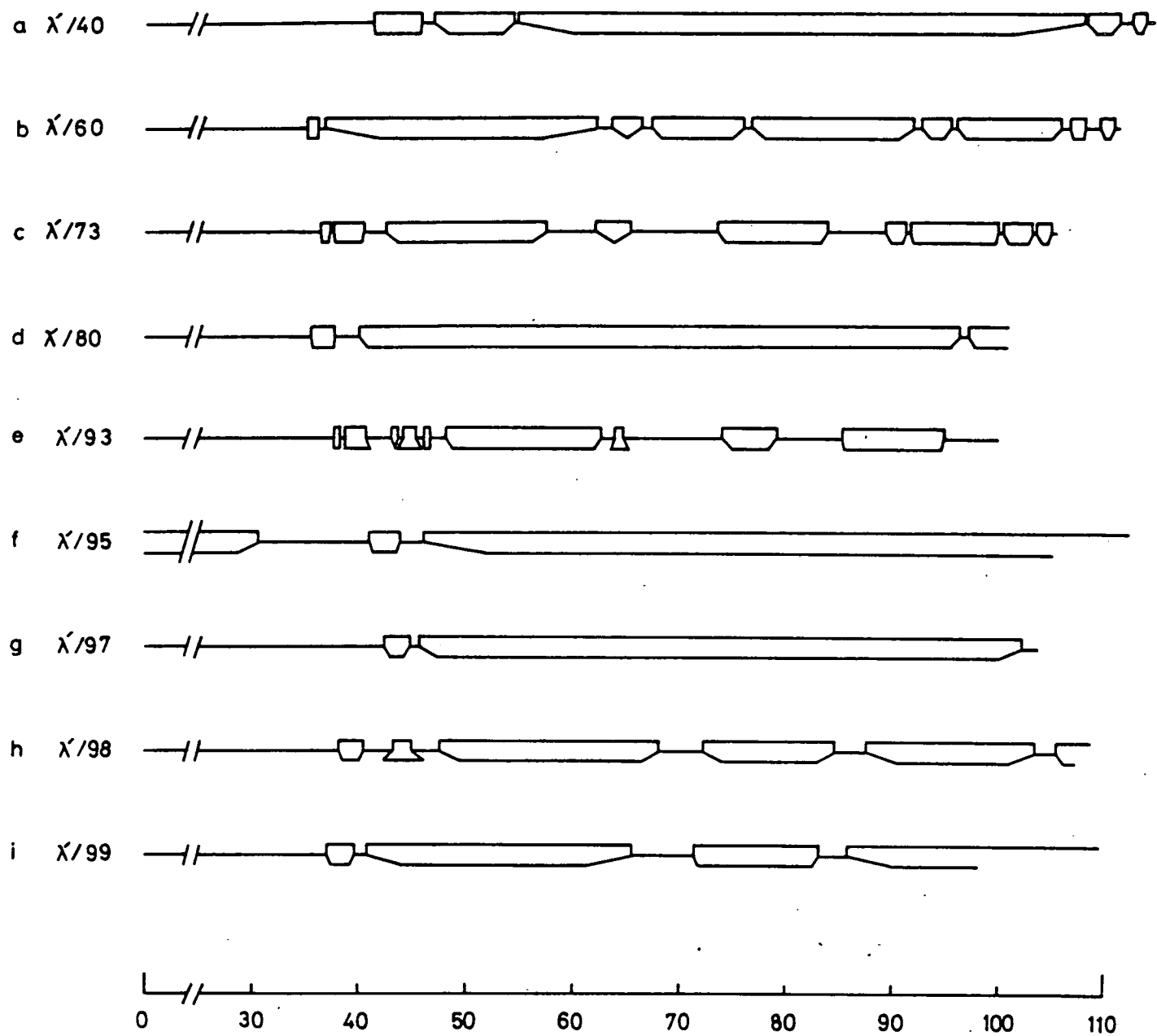
Figure 2.1 Heteroduplexes between the DNA molecules of phage λ and nine newly isolated phages. (Single strands not assigned.)
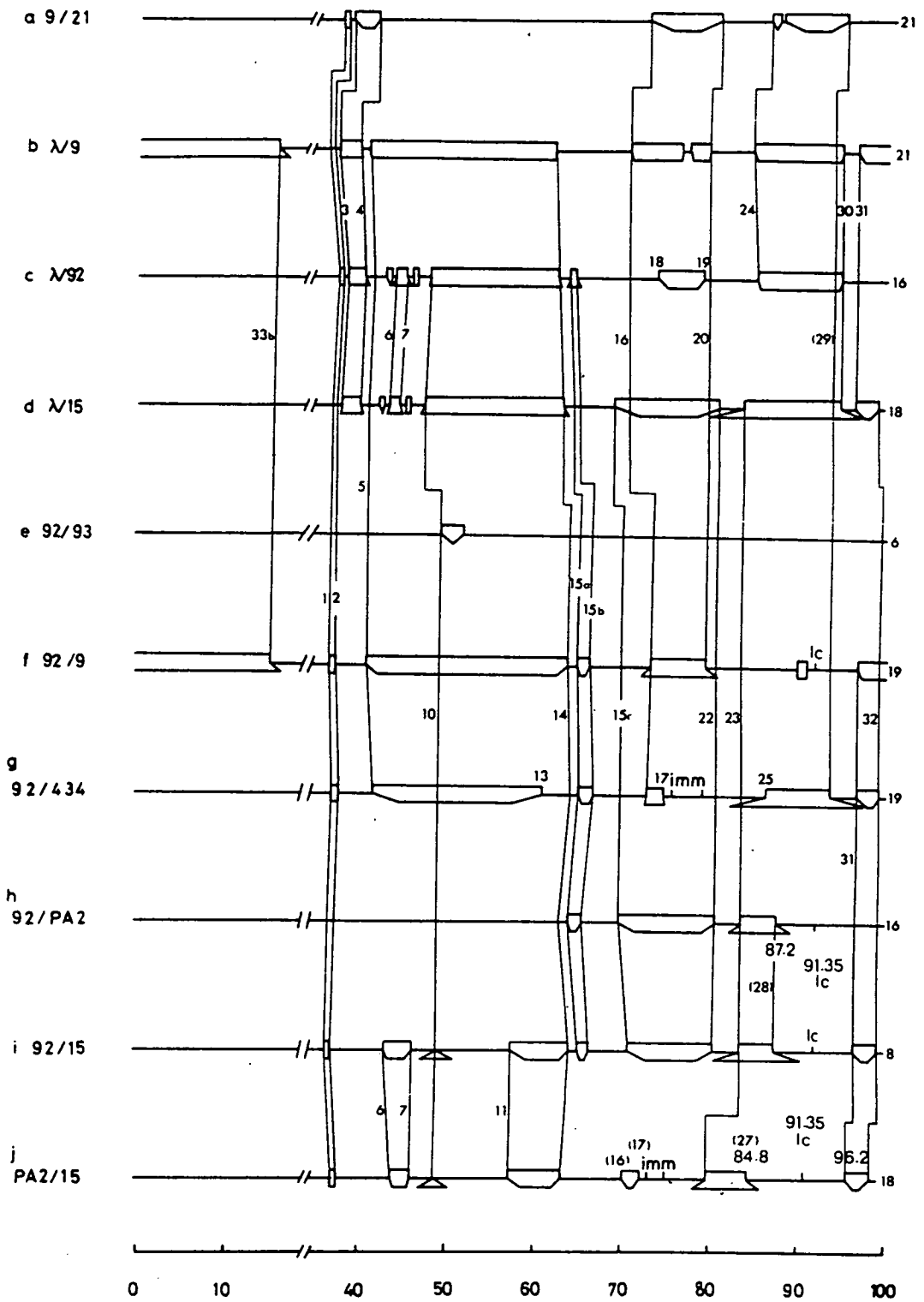
Figure 2.2 Heteroduplexes between the phage 9, 15, and 92 (new) and the phage λ, 21, 434 and PA2 (old) DNA molecules. (The number of molecules measured is given on the right; boundary positions are in Appendix 2.1; mode of assignment of single strands is in Appendix 2.2.)

Figure 2.3 Heteroduplexes between the phage 15, 28, 84A, 84B and 92 (new) and the phage λ, λp4, 21, 82 and PA2 (old) DNA molecules. (The number of molecules measured is given on the right; boundary positions are in Appendix 2.1; mode of assignment of single strands is in Appendix 2.2.)

that the genomes of the new phages could be divided into the same segments as the old phages (Table 2.6). Only in segment 15, was an extra segment defined. Phages 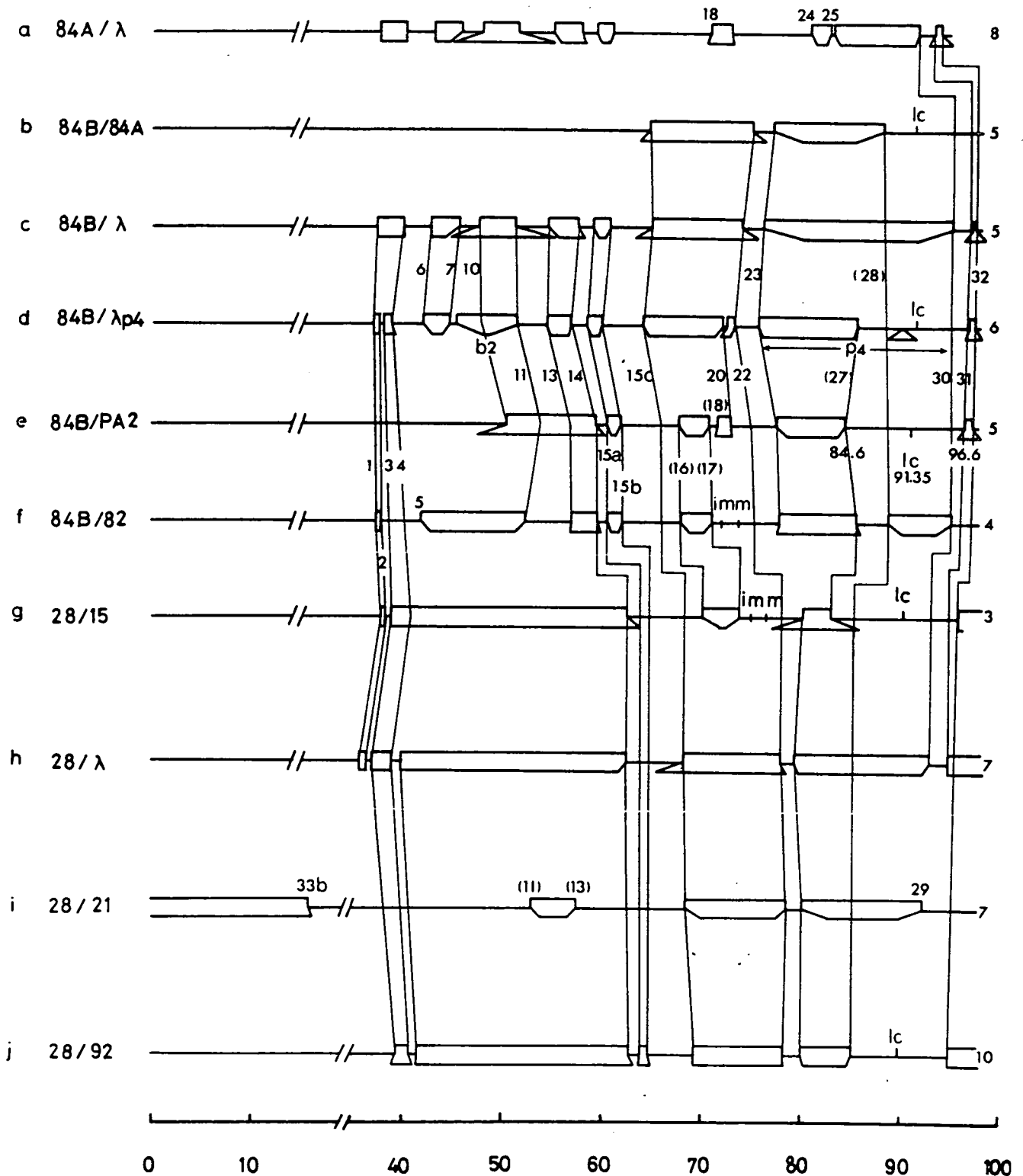92, 84A and 84B had a new allele within this segment. So segment 15 was divided into three segments, named 15a, 15b and 15c. Segment 15b is the one newly defined. In the six 'old' phages there were two segments, 17 and 19, which had a different allele in each phage (Table 1.3). However, in segment 17, the DNA molecules of phages 92 and 84A are homologous with that of λ. In segment 19, the immunity region, phage 92 is homologous with phage 434, phages 15 and 28 are homologous with PA2, phage 84B is homologous with phage 82. Thus, there is no segment that has a different allele in each of these twelve phages.

To look for the combinations of alleles indicating exchange, the useful segments are only the ones for which two alleles occur more than once. They must be defined segments, preferably of known function. All such segments are shown in Table 2.7. In each segment, alleles which occur only once are omitted.

Twelve different types of combination of alleles indicating exchanges between phage genomes were found. They are listed in Table 2.8. Every type of combination of alleles occurs in more than one group of four phages. (Only two combinations in fact involve the immunity segment.)

The combination of longest segments for which the functions are known involves phages 9, 21, 28 and 92 (Figure 2.4a). In segment 33b, which includes the head and tail genes, 9 and 21 are homologous and

Table 2.6 The allelic[a] Composition of the twelve phage genomes

Segment number (same as right boundary[b] number)

Phage	33b	1	2	3	4	5	6	7	8	9	10	11	13	14	15a	15b	15c
λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ
434	λ	λ	λ	λ	434	λ	434	434	434	434	434	434	λ[e]	434	λ	λ	λ
82	λ	λ	λ	λ	434	λ	434	434	434	434	434	434	λ[e]	434	λ	λ	λ
PA2	λ	λ	PA2	λ	434	λ	λ[c]	PA2	λ	PA2	λ	PA2	PA2	434	λ	λ	λ
21	21	λ	PA2	λ	21	λ	21	21	21	21	21	21	21	21	λ	λ	λ
424	λ	λ	PA2	λ	434	λ	λ[c]	424	λ	424	λ	424	424	424	424	424	424
92	λ	λ	PA2	λ	434	λ	λ	PA2	λ	PA2	λ	PA2	PA2	434	λ	92	λ
9	21	λ	λ	λ	434	λ	21	21	21	21	21	21	21	21	λ	λ	λ
15	λ	λ	λ	λ	434	λ	λ	15	λ	PA2	λ	PA2[d]	15	15	λ	λ	λ
84A	λ	λ	PA2	λ	434	λ	λ[c]	PA2	λ	PA2	λ	84A	λ	84A	λ	84A	λ
84B	λ	λ	PA2	λ	434	λ	λ[c]	PA2	λ	PA2	λ	84A	λ	84A	λ	84A	λ
28	λ	λ	PA2	λ	21	λ	21	21	21	21	21	21	28	21	λ	λ	λ

	16	17	18	19	20	22	23	24	25	26	27	28[f]	29	30	31	32	33a
λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ	λ
434	λ	434	λ	434	λ	λ	λ	λ	434	λ	λ	434	434	434	λ	434	λ
82	82	82	82	82	82	82	λ	82	82	82	82	82	82	λ	λ	434	λ
PA2	82	PA2	82	PA2	PA2	82	λ	PA2	PA2	PA2	PA2	82	PA2	434	λ	λ	λ
21	λ	21	21	21	21	λ	λ	λ	434	λ	21	21	21	434	λ	434	21
424	424	424	424	424	424	424	424	424	424	424	424	424	434	λ	λ	λ	λ
92	λ	λ	λ	434	λ	λ	λ	λ	434	92	92	92	PA2	434	λ	λ	λ
9	λ	9	9	9	9	λ	λ	λ	434	92	92	92	PA2	434	λ	434	21
15	82	15	82	PA2	PA2	82	λ	15	15	15	15	82	PA2	434	λ	434	λ
84A	λ	λ	λ	84A	λ	λ	λ	λ	84A	84A	84A	84A	PA2	434	λ	434	λ
84B	82	84B	82	82	82	82	λ	84B	84B	84B	84B	82	PA2	434	λ	434	λ
28	82	28	82	PA2	PA2	82	λ	28	28	28	28	82	PA2	434	λ	434	21
λp4								82	82	82	82	82	PA2[g]	434			

a. Alleles given as '9''28''15', '92', '84A', '84B' are not necessarily different from the other as all the possible heteroduplexes have only been made between the first six genomes.

b. Solid lines define boundaries which can be related to positions in the λ molecule. Broken lines define boundaries which can be related to oneanother but not to positions in the λ molecule.

c. 0.6% deleted relative to λ.

d. 3.6% inserted relative to PA2.

e. 0.9% inserted relative to λ.

f. 92 is non-homologous with 9 for part (1%) of this segment.

g. 2.5% inserted relative to PA2.

Table 2.7 Segments for which two alleles occur more than once

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Segment number

Phage	33b	2	4	11	13	6-14	15b	19	22	32	33a
λ	λ	λ			λ	λ	λ		λ	λ	λ
434	λ	λ	434		λ	434,λ	λ	434	λ	434	λ
82	λ	λ	434		λ	434,λ	λ		82	434	λ
PA2	λ	PA2	434	PA2	PA2	λ,PA2,434	λ	PA2	82	λ	λ
21	21	PA2	21			21	λ		λ	434	21
424	λ	PA2	434							λ	λ
92	λ	PA2	434	PA2	PA2	λ,PA2,434		434	λ	λ	λ
9	21	λ	434			21	λ		λ	434	21
15	λ	λ	434				λ	PA2	82	434	λ
84A	λ	PA2	434	84A	λ		84A		λ	434	λ
84B	λ	PA2	434	84A	λ		84A		82	434	λ
28	λ	PA2	21			21	λ			434	21

Table 2.8   Combinations of alleles

indicating exchanges between genomes

	Segment	number
Phage	-------------------------------------	
	2 (J)	22 (O)

9, 434, λ	λ	λ
15, 82	λ	82
84A, 92, 21	PA2	λ
28, 84B, PA2	PA2	82

| | 2 (J) | 19 (imm) |

434	λ	434
15	λ	PA2
92	PA2	434
28, PA2	PA2	PA2

| | 2 (J) | 32 (136) |

λ	λ	λ
15, 9, 82, 434	λ	434
92, 424, PA2	PA2	λ
28, 84A, 84B, 21	PA2	434

Table 2.8 (continued)

---

	2 (J)	33 (Head)
15; 82, 434, λ	λ	λ
9	λ	21
28(33a),84A,84B,92,424,PA2	PA2	λ
28(33b), 21	PA2	21

---

	13 (int, xis)	22 (0)
84A, 434, λ	λ	λ
84B, 82	λ	82
92	PA2	λ
PA2	PA2	82

	15b (exo)	22 (0)
9, 21, 434, λ	λ	λ
28, 15, PA2, 82	λ	82
84A	84A	λ
84B	84A	82

Table 2.8 (continued)

---

	22 (0)	32 (136)
92, λ	λ	λ
84A, 9, 21, 434	λ	434
PA2	82	λ
28, 84B, 15, 82	82	434

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	19 (imm)	32 (136)
434	434	434
28, 15	PA2	434
92	434	λ
PA2	PA2	λ

	33b (Head)	4 (Host spec.)
9	21	434
21	21	21
92	λ	434
28	λ	21

Table 2.8 (continued)

---

	4 (Host spec.)	22 (O)
21	21	λ
28	21	82
84A, 9, 92, 434	434	λ
84B, 15, PA2, 82	434	82

	11(Ea47,Ea31,Ea59)	22 (O)
PA2	PA2	82
92	PA2	λ
84B	84A	82
84A	84A	λ

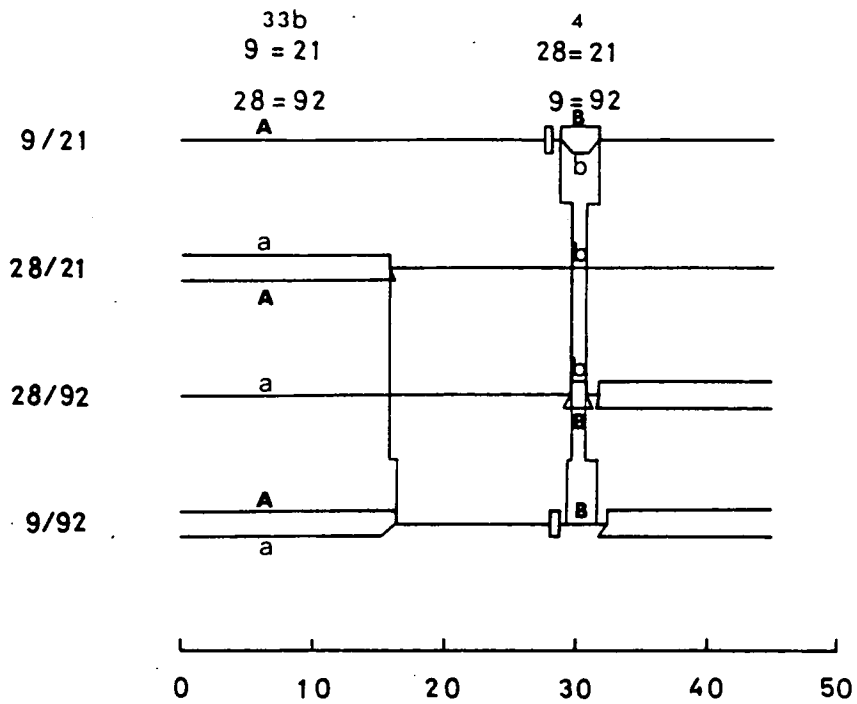|	6-14	22 (O)
21, 9	21	λ
28	21	82
(434), 92	λ,PA2,434,(434,λ)	λ
(82), PA2	λ,PA2,434,(434,λ)	82

Figure 2.4a Evidence for exchange of segments between genomes by recombination. The segments are lettered to fit Figure 2.4b.

28 and 92 are homologous; in the other segment, segment 4, which determines host specificity, 9 and 92 are homologous and 21 and 28 are homologous. These four phages can be fitted into Campbell's diagram (Figure 2.4b) which shows that they cannot have evolved from a common ancestor without exchanging segments.

The diagram in Figure 2.4b assumes that 'homologous' regions in the heteroduplexes are entirely homologous, and that since non-homologous regions differ significantly it is impossible for the A to a or B to b transition to occur twice independently because of the large number of identical mutations which would be necessary. In an attempt to measure the actual degree of homology in segments 33b and 4 the 28/21, 28/92, 9/21 and 9/92 heteroduplexes were spread in high concentrations of formamide as described by Davis and Hyman (1971). The λ alleles of segments 33b and 4 have a GC content of about 55% (Sanger et al, 1982), and electron microscopic denaturation studies (Highton, unpublished) indicated that the 21 alleles had a similar GC content. Since the molecules shown as non-homologous in these segments in Figure 2.4b appeared thus in a hyperphase of 50% formamide then they differed in sequence by at least 18%, if they were 55% GC. With a hyperphase containing 70% formamide the DNA molecules shown as identical in segments 33b and 4 still appeared homologous. This means that they differed by less than 9%. However, referring back to Figure 2.4b, even if the second 'forbidden' transition produced an allele only 91% the same as the first it would still require

$$\frac{(18-9)}{18} = \frac{1}{2}$$

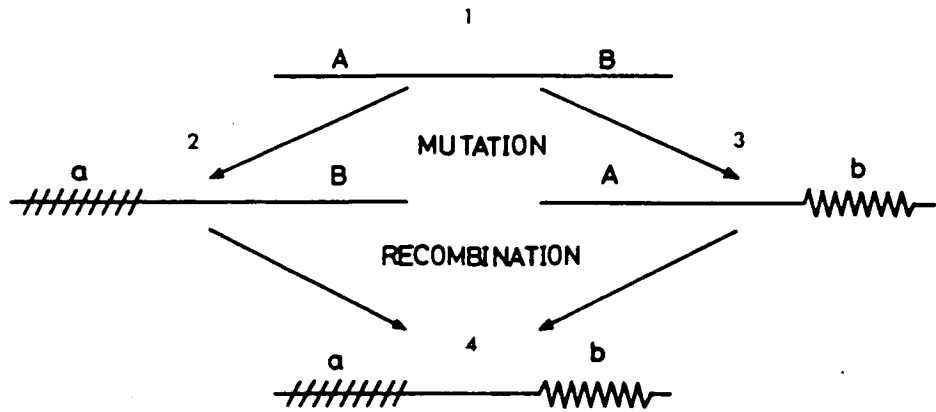of the mutations to be identical. If in the first A to a transition

Figure 2.4b (After Campbell, 1977) Genomes (2) and (3) can be generated

from (1) by mutation, but (4) can only be produced by recombination

unless the A to a or B to b transitions occur twice.

18% of the base are changed, then the probability that a mutation in the second transition would occur at the same place as one in the first, would be 18/100, and the probability that the mutation would also be of the same type would be

$$\frac{18}{100} \times \frac{1}{3} = \frac{1}{16}$$

For a length of n bases, $n \times 9/100$ of the mutations would need to be the same as in the first transition. The probability of this would be,

$$\left(\frac{1}{16} \right)^{n \times 9/100}$$

Having the twelve combinations of alleles indicating exchanges as evidence, the conclusion can be made that the possibility of the lambdoid phages having developed by linear evolutionary divergence alone, without exchange of segments, from a common ancestor which contained base sequences ancestral to all those of the existing phages is eliminated.

DISCUSSION

Among 117 samples, although only 33 phages were isolated, more phages were detected. In the third batch of samples, every sample was found to harbour one or more phages. We can assume that the other batches contained similar numbers of phages. With the system used to isolate phages, further phages may have been missed because of different host ranges. For example, strain TGL70 is tonA, which is resistant to the phages ϕ80, T1 and T5 (Braun and Hantke, 1977).

I was able to make lysogens of C600 from most of the phages. UV induction tests showed that more than half of them were UV inducible. These UV inducible phages all share homology with λ. This suggests that lambdoid phages exist widely in nature. There might be at least one or two of them inside the body of every human being and probably of every animal which harbours E.coli.

During the immunity test, it was found that phages 18 and 40 grew very well on C600(λ), but did not grow at all on C600 and other lysogens of C600 (Table 2.3). Further phages from 4 samples only grew on C600(434), C600(82) and C600(424). It was thus not possible to isolate phages from these samples. There must have been some changes which occurred to the cells because of harbouring the prophages. Temperate phages have been found to confer characteristics on the lysogenic cell which are not obviously pertinent to lysogeny. This phenomenon is called lysogenic conversion. A common type of lysogenic conversion is the modification of surface antigens of the host cell.

The changes observed above may also be cell surface changes. The receptor on the C600 surface may be switched to another type after lysogenization by some phages.

Many phages related to λ in some way, e.g., serologically, by ability to recombine with λ or by DNA homology, have been isolated (Jacob and Wollman, 1956; Bertani, 1971; Matsushiro, 1963; Parkinson, 1971; Rock, 1974; Schnaitman et al, 1975; Tsygankov et al, 1976; Schleuderberg et al, 1980; Dhillon et al, 1980). The DNA molecules of those which have been compared by the electron microscopic heteroduplex technique, namely 434, 424, 82, 21, PA2, Φ80 and Φ81 all show large regions of non-homology with one another, and with λ (Simon et al, 1971; Fiandt et al, 1971; Niwa et al, 1978; Highton et al, unpublished). The heteroduplexes in Figures 2.1, 2.2 and 2.3 show that a further fifteen newly isolated phages are quite different from one another and from the seven above. The structure of the DNA molecules of six of the new phages was studied in detail (Figures 2.2 and 2.3) and it was shown that they could be divided into the same segments as the six old phage DNA molecules analysed in Section 1 (Table 2.6). Thus the population which was sampled may consist of large number of different phages, with genomes composed of (almost) any combination of the possible alleles for each segment. Further, since the phages isolated at different times have never been found to have completely homologous genomes, it may be that the population is constantly changing by exchange of segments. Campbell and Botstein (1983) have argued that the available genetic and physical data on the structure of lambdoid phage genomes indicate that they have evolved from a common ancestor. The results summarised above indicate that exchange of

segments has occurred at sometime during their evolution, and therefore may still be occurring, possibly frequently. Recombinants between phages containing homologous regions can be readily constructed in the laboratory, and spontaneous recombinations between phages and defective prophages in the E.coli genome have been detected. Three defective prophages on the E.coli K-12 chromosome have been reported (Kaiser and Murray, 1979; Kaiser, 1980; Espion et al, 1983). Phages λp4 (Jacob and Wollman, 1954) and λrev (Zissler et al, 1971) were produced by recombination with the qsr' and rac prophages respectively (Kaiser, 1980; Kaiser and Murray, 1979). There might be further prophage-like pieces of DNA in K-12 and other E.coli strains. A stock of phage 21 grown from a C600 lysogen obtained from Dr. E. Wollman was found to consist largely of a variant of phage 21. This new phage is homologous with phage 21 apart from the immunity region and the QSR region. It is very likely that phage 21 exchanged part of its genome with the host chromosome. It contains the ic gene (see Section 3), and probably the qsr' genes, of the qsr' prophage. Phages φ80 and φ81 were isolated from one cell (Matsushiro, 1963). They are non-homologous in the region from 48% to 83%. It could be that one mother phage infected the cell and then a daughter phage was generated by exchange with the cell chromosome. Newly isolated phages 84A and 84B were isolated from one sample. They are homologous except the regions from 64.4 to 74.6%λ and from 76.8 to 87.8%λ, a total of 21.2%λ. One of these two phages might also have been generated from the other by exchange with the host chromosome.

Further evidence in favour of the evolution of the various genomes from a common ancestor, not mentioned by Campbell and Botstein

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(1983), is that the amino acid sequences of the λ and 434 <u>cro</u> proteins show significant homology and so do those of the <u>cI</u> proteins (Sauer <u>et al</u>, 1982). Also, as cited in Section 1, Yocum <u>et al</u> (unpublished) found some regions of more than 50% homology between the base sequences of λ and 434 <u>cI</u> genes. Campbell and Botstein (1983) also speculated that the ancestral common genome may, at least in part, have been derived from the <u>E.coli</u> genome. Consistent with this the <u>Lac</u>, <u>Gal</u> and <u>LexA</u> repressors of <u>E.coli</u> were found related to the phage repressors (Sauer <u>et al</u>, 1982). They have similar structure and share sequence homology with the phage repressors. These <u>E.coli</u> repressors and their binding sites might be the source of the different phage immunities. Similarly, other <u>E.coli</u> genes which have the same function as phage genes may still serve as a source of new phage genes. Recombinations between phage and bacterial genomes may occur readily at a low frequency, which is hardly detectable during normal infection. Whenever selective pressure is applied, the recombinants which have evolutionary advantages will survive and propagate. Hence the new phage strain appears.

Campbell and Botstein (1983) have argued that "the unit of selection is not the phage but some lesser functional unit" and that "in such a theory, the product of evolution is not a given phage but, instead the family of phage with interchangeable genetic elements." Kaiser (personal communication) has argued that since most naturally occurring <u>E.coli</u> that have been studied appear to be lysogenic for a lambdoid prophage, or defective prophage, the strongest selective pressure may be for diversity which allows a phage to infect a cell lysogenic for a different phage. This idea was based on the number of alleles found

to occur for each segment of the genomes of the six phages analysed in Section 1. Figure 1.3 and Table 2.6 show that this idea is supported by the six new phages. Segments with greatest diversity are those in which a difference from a resident prophage would facilitate infection.

Appendix 2.1   The positions of the boundaries in the heteroduplexes in Figure 2.1

Heteroduplex   Boundaries

Figure 2.1

a. $\lambda'/40$ 41.6 46.0 47.1 54.6 54.9 108.0 108.4 111.3 112.7 113.9 114.6
          41.6 45.9 47.0 52.0 52.3 93.6 94.0 95.6 97.0 97.5 98.2

b. $\lambda'/60$ 35.4 36.4 37.1 62.3 63.8 66.7 67.6 76.0 76.8 92.1 92.8 95.4 96.1 106.0 106.8 108.3 109.5 110.9 111.5
          35.4 36.4 37.0 52.1 53.5 -   54.4 61.1 61.8 74.6 75.4 76.6 77.3 85.5 86.4 87.8 89.0 89.6 90.1

c. $\lambda'/73$ 36.7 37.5 37.9 40.7 42.8 57.7 62.2 65.6 73.8 83.9 89.5 91.3 91.8 100.0 100.5 103.3 103.6 105.1 105.6
          36.7 37.4 37.8 40.4 42.6 54.7 59.2 -   67.4 76.6 82.2 83.3 83.7 91.7 92.2 94.1 94.4 94.7 95.3

d. $\lambda'/80$ 35.8 38.0 40.2 96.3 97.2 101.0
          35.8 38.0 40.2 94.8 95.8 99.0

e. $\lambda/93$ See Figure 2.2e. 93 differs from 92 only by a deletion of 2.4% at 49.5% in 92.

f. $\lambda'/95$ 30.6 41.0 43.9 46.1 112.2
          28.8 39.2 41.7 43.9 97.2

g. $\lambda'/97$ 42.5 44.9 45.7 102.1 103.7
          42.5 43.7 44.5 97.3 98.9

h. $\lambda'/98$ 38.3 40.6 43.5 45.0 47.6 68.1 72.2 84.5 87.6 103.4 105.4 108.8
          38.3 40.2 43.1 46.8 49.4 66.4 70.4 79.5 82.6 93.2 95.2   96.3

i. $\lambda'/99$ 37.2 39.7 40.9 65.6 71.5 83.1 85.9 109.5
          37.2 39.0 40.2 57.5 63.3 74.1 76.9 85.1

Appendix 2.2   The Positions of the Boundaries in the Heteroduplexes in Figures 2.2, 2.3 and 3.5

Heteroduplex	33b	1	2	3	4	5	6	7	8	9	10	11	13	14	15a	15b	15c	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33a
											Boundary →							← **Number**																	
Figure 2.2																																			
a. 9/21		37.7	38.3	38.8	41.7														72.3				80.3			86.1	87.5				94.8				100.4
		37.7	38.3	38.8	40.0														70.6				73.9			79.6	80.1				83.4				89.0
b. λ/9	15.6			37.8	40.1	41.1								62.1					70.8				79.7			84.8						95.0	96.7		100.0
	16.9			39.0	41.7	42.7								63.8					72.4				80.8			86.0						95.7	97.4		100.4
c. λ/92		37.7	38.4	38.8	40.8		44.3	45.4	46.1	46.7	48.2			62.8	64.0	64.8								74.0	79.1	85.2					94.9				100.0
		37.7	38.4	38.8	41.1		44.0	46.6	47.3	47.9	49.4			64.4	65.7	67.3								76.6	80.1	86.1					95.5				100.6
d. λ/15				38.2	40.2		43.6	44.7	45.4	46.0	47.6			63.4			69.2							81.0	83.8							95.0	96.5	99.2	100.0
				38.2	40.6		43.4	45.2	45.9	46.4	48.1			65.0			70.7							76.8	79.6							97.0	98.5	99.3	100.0
f. 92/9	15.2	37.0	37.7		41.3									64.1	65.2	66.6		73.6				80.0											97.3		100.6
	16.4	38.2	38.9		42.5									62.8	63.9	64.6		71.6				80.1											97.5		100.4
g. 92/434		37.4	38.1		42.0								61.3		65.5	67.1		73.2	75.0								86.8				94.2		97.2	99.8	100.6
		37.4	38.1		42.0								54.9		59.1	59.9		66.0	68.2								80.0				95.5		98.5	99.3	100.0
h. 92/PA2														64.4	66.0		70.2							81.2	84.1					88.2					100.6
														64.4	65.2		69.3							77.2	80.1					87.2					99.6
i. 92/15		36.8	37.4					43.5	46.6				49.4	58.0	64.6	65.6	66.9	71.3							81.0	84.0					88.0			97.1	99.8	100.6
		36.8	37.4					43.5	45.5				48.2	60.6	64.6	65.6	66.2	70.6							76.4	79.4					89.5			98.6	99.3	100.1
j. PA2/15		37.4	38.0					44.2	46.4				49.1	57.6	63.6				70.8	72.7						80.1				84.8				96.2	98.8	99.6
		37.4	38.0					44.2	45.9				48.6	60.6	64.3				71.5	72.0						79.5				87.3				98.7	99.3	100.0
Figure 2.3																																				
a. 84A/λ		37.5			40.1	42.8	45.7					47.7	51.3	54.7	57.6	59.1	60.7			70.4							80.4	82.4						91.3	93.0	93.6	94.3
		37.5			40.2	42.9	44.4					46.5	56.7	60.1	62.8	64.3	65.1			74.8							85.4	86.6						94.8	96.5	99.2	100.0
b. 84B/84A																	64.4							74.6	76.8					87.8					97.9
																	64.4							77.0	79.3					84.2					94.3
c. 84B/λ		37.3			39.9	42.6	45.5					47.5	51.1	54.4	57.3	58.8	60.5	64.7							73.7		75.9						94.9	96.9	97.1	97.9
		37.3			40.0	42.7	44.2					46.2	56.5	60.1	62.5	64.0	64.8	69.0							81.3	83.5						94.9	96.9	99.2	100.0
d. 84B/λp4		37.0	37.5	38.0	38.8		42.0	44.6	45.3				51.3	54.3	56.6	58.3	59.7	63.8							71.9	72.2	73.0	75.4		85.3	90.0			96.7	97.2	97.9
		37.0	37.6	38.1	39.5		42.7	43.8	44.5				45.1	48.0	50.1	51.8	52.6	56.7							63.4	63.7	64.8	67.2		76.4	81.0			90.8	92.6	93.3
e. 84B/PA2												50.3		59.2	60.4	61.6		67.5	70.5	71.4				72.6		77.3			84.3				96.4	97.1	97.9
												50.3		63.0	64.2	64.8		70.7	72.5	73.4				74.9		79.5			84.6				96.6	98.8	99.6
f. 84B/82		37.3	37.8		41.8							(52.2)	56.8	59.4	60.4	61.9		67.8	70.9						77.6				85.4	88.6		95.0			97.9
		37.3	37.8		41.8							(50.3)	54.9	58.0	59.0	59.7		·65.6	67.0						73.7				82.1	85.3		88.6			91.5
g. 28/15		37.7	38.3	38.8										62.4				70.0	73.7						80.1*			82.9						95.8	98.1
		37.7	38.3	38.8										63.6				71.2	71.5						77.8			86.6						99.4	100.1
h. 28/λ		35.8	36.6	37.0	39.1	40.1								62.3			68.2							78.0	79.5*							93.1	94.9		98.0
		35.8	36.6	37.0	39.1	40.1								61.4			67.3							80.3	81.8							94.4	96.2		100.0
i. 28/21	15.8											53.1	57.6				68.5							78.6	80.1*						92.4				98.0
	16.0											53.3	56.3				67.2							74.9	76.4						83.4				89.0
j. 28/92				39.6	40.8	41.8								62.9	64.1	64.9	69.4							78.4	80.1*				85.3					95.1	98.0
				39.6	41.9	42.9								64.7	65.9	67.6	72.1							81.3	83.0				87.5					97.3	100.6
Figure 3.5																																				
λp4/PA2		38.3	39.0	39.5	41.5		45.0	46.0	46.7				52.1				58.1							65.1	68.2				75.9						93.3
		38.3	39.0	39.5	41.8		44.8	47.1	47.8				63.0				69.0							76.6	79.7				84.7						99.6

* These positions are about 1% to the left of the boundary 23.

Appendix 2.3 Assignment of Strands in the Non-homologous Regions

in Figures 2.2, 2.3 and 3.5

Heteroduplex	Regions	Heteroduplexes used for assignment

Figure 2.2

a. 9/21 $\lambda/21^a$ and $\lambda/9$

b. $\lambda/9$ 4-30 $\lambda/21^a$ and $9/21^b$

                    32, 33 $\lambda/21^a$ and 9/21 (homologous)

c. $\lambda/92^g$ 1-14 $\lambda/PA2^a$ and 92/PA2 (homologous) and λ' insert^c

                    19         $\lambda/434^a$ and 92/434 (homologous)

                    25-30 $\lambda/9$ and 92/9 (homologous)

d. $\lambda/15$ 4 $\lambda/92$ and 92/15 (homologous)

                    7          $\lambda/92$ and 92/15

                    10-14 $\lambda'/15$ and $\lambda/15^d$

                    16-32 $\lambda/92$ and $92/15^b$; $\lambda/PA2^a$ and $PA2/15^b$

e. 92/93 $\lambda/92$ and $\lambda/93$

f. 92/9   16-14 $\lambda/92$ and $\lambda/9$; 92/PA2 (homologous) and

                       9/21 (homologous) and $PA2/21^a$

            17-33 $\lambda/92$ and $\lambda/9$

g. 92/434 $\lambda/92$ and $\lambda/434^a$

h. 92/PA2 $\lambda/92$ and $\lambda/PA2^a$; 92/434 and $434/PA2^a$

i. 92/15   λ/92 and λ/15

j. PA2/15   λ/PA2^a and λ/15; 92/PA2 and 92/15

Figure 2.3

a. 84A^g/λ   2-15b   84B/84A^e (homologous) and 84B/λ

                     15c-30   84B/84a^e and 84B/λ

b. 84B/84A   84B/λ^e and 84A/λ

c. 84B/λ   2-10   84B/PA2 (homologous) and λ/PA2^a

                     11   84B/λp4 (b2 deletion)

                     15c-30   84B/84A^e and 84A/λ

                     32   84B/82 (homologous) and λ/82

d. 84B/λp4   84B/λ and λ/λp4^f

                     15-22   84B/82^b and 82/21^a

e. 84B/PA2   84B/λ and λ/PA2^a

f. 84B/82   84B/λ and λ/82^a

g. 28/15   λ/15 and 28/λ^b

h. 28/λ   λ/15 and 28/15^b

i. 28/21   λ/21^a and 28/λ

j. 28/92   28/λ and λ/92; 28/15 and 92/15

Figure 3.5

λ/p4/PA2   λ/λp4^f and λ/PA2^a

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a.   See Figure 1.2.

b.   Three heteroduplexes can be made from three molecules. If the strands are assigned in one, then they can also be assigned in any region in the other two which can be shown to correspond to a region in the assigned molecule.

c.   All the 'λ' heteroduplexes were made with λ' DNA which contains an insertion of 2.7% at 61%. This is not included in Figures 2.2 and 2.3.

d.   The λ/15 heteroduplex was made with both λ andλ' DNA.

e.   Only one way of assigning the strands in 84A/λ, 84B/λ and 84B/84A gives consistent lengths in this region.

f.   See Figure 1.5a.

g.   The longer strand of segment 15b has been assigned to 92 and 84A to give the best total length agreement.

SECTION 3. LYSOGENIC CONVERSION OF E.COLI K-12 BY LAMBDOID PHAGES.
EVIDENCE THAT GENE NMPC IS ON THE DEFECTIVE QSR' PROPHAGE.

INTRODUCTION

The outer membrane of Gram-negative bacteria lies outside the cytoplasmic membrane and peptidoglycan layer and forms the interface between the cell and its external environment. It is composed of proteins, lipopolysaccharide and phospholipids. It is known to function as a barrier to antibiotics, detergents, and other toxic chemicals, also, it has permeability properties that allow entry of nutrient solutes from the medium. In addition, the components of the outer membrane function as receptors for bacteriophages and bacteriocins.

The outer membrane of E.coli and many other Gram-negative bacteria contains a group of major outer membrane proteins. These proteins have a molecular mass in the range of 35,000 to 45,000 Daltons and many make up as much as 70% of the total outer membrane proteins (Schnaitman et al, 1975).

Identification and genetic analysis of the major outer membrane proteins of E.coli has been carried out in a number of laboratories. The most effective means of separating these major outer membrane proteins is with the SDS alkaline gel electrophoresis system described by Bragg and Hou (1972), or with the discontinuous buffer gel systems of the types described by Neville (1971) or Laemmli (1970).

The outer membrane of E.coli K-12 has 3 major proteins, OmpF (1a), OmpC (1b) and OmpA. OmpF and OmpC act as porins (Osborn and Wu, 1980), they facilitate the passage of a variety of low molecular weight soluble molecules through the outer membrane (Nakae, 1976). OmpF is known to be the receptor for phages TuIa (Datta et al, 1977) and T2 (Hantke, 1978). OmpC is the receptor for phages PA2 (Bassford et al, 1977), 434 (Hankte 1978), TuIb (Datta et al, 1977) and Mel (Verhoef et al, 1977), it also plays a role in the receptor function for phage T4 (Yu and Mizushima, 1982). Genes ompF and ompC are the structural genes for these two proteins (Hall and Silhavy, 1979; Mutoh et al, 1981). Another locus, ompB (Sarma and Reeves, 1977; Verhoef et al, 1979), acts as a positive regulatory gene at the transcriptional level (Hall and Silhavy, 1979; Hall and Silhavy, 1981).

It was shown recently that the synthesis of the OmpF protein is effected by the tolC locus, probably at a post-transcriptional control level (Morona and Reeves, 1982). OmpA is a transmembrane protein; it acts as a receptor for phages TuII[*] (Henning and Haller, 1975; Henning et al, 1976) and K3 (Manning et al, 1976; van Alphen et al, 1977). It functions directly in promoting formation or maintenance of mating pairs (Osborn and Wu, 1980), and it plays a significant role in maintenance of cell morphology and outer membrane integrity (Osborn and Wu, 1980). The ompA gene is the structural gene for this protein (Bachmann and Low, 1980; Reeves, 1979).

Temperate phages have been found to confer characteristics on the lysogenic cell which are not obviously pertinent to lysogeny. This phenomenon is called lysogenic conversion. A common type of lysogenic

conversion is the modification of surface antigens of the host cell. Lysogenic conversion often involves exclusion of superinfecting phages, or resistance to phages. For λ, it is known that it confers on the host bacteria the capacity to block the multiplication of rII mutants of T4 (Dove, 1971) and also resistance to complement (Saunders, 1981). Different types of lysogenic conversion caused by E.coli phages, P1 and P2 and Salmonella phages P22 and ε15 have been reported (Dove, 1971).

Another example of lysogenic conversion by a lambdoid phage was reported by Schnaitman et al (1975). An E.coli K-12 strain which had been mated with a pathogenic E.coli strain isolated from a pig was found to produce a new protein named 2, and a temperate bacteriophage was isolated from this K-12 strain after induction with UV light. This phage, named PA2, was a lambdoid phage. It was identical with λ in morphology and hybridized readily with λ. When strains of E.coli K-12 were lysogenized by phage PA2, they acquired the ability to produce protein 2, and the production of protein 1, later shown to consist two proteins 1a and 1b (OmpF and OmpC), was greatly reduced. Protein 1 was the receptor for PA2, the mutants lacking protein 1 were resistant to PA2. Protein 2, the product of a gene of phage PA2, was also a porin (Pugsley and Schnaitman, 1978a). It was not a receptor of PA2. Adsorption of PA2 to lysogenic strains of PA2 was significantly reduced compared to non-lysogenic strains.

Mutant strains producing new outer membrane proteins which are not present on the surface of wild type cells were selected in strains lacking proteins OmpF and OmpC. These proteins, NmpA, NmpB and NmpC, which appeared to function as pores were produced as a result

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of mutations at three loci designated as nmpA, nmpB and nmpC respectively (see Osborn and Wu, 1980). The proteins NmpA and NmpB appeared to be identical by peptide analysis and isoelectric focusing. The NmpC protein, which is quite different from these and from the OmpC and OmpF proteins, is closely related structurally and immunologically to protein 2 produced by PA2. Only one different peptide was observed on comparing the proteolytic peptide maps of the NmpC protein and of protein 2, and there were only slight differences in the isoelectric focusing profiles. Antiserum against protein 2 showed partial cross-reactivity with the NmpC protein. Very likely, the genes for these two proteins are from the same ancestral gene, and there has been some limited divergence of the genes. The ancestor of phage PA2 possibly acquired the gene by recombination with the E.coli chromosome (Lee et al, 1979).

The gene of the phage PA2 which determines the protein 2 production has been designated as the lc gene (for lysogenic conversion). Recombination with phage λ indicated that the lc locus was on the right arm of the PA2 genome, near the QSR region (Gregg and Schnaitman, unpublished). Further, they obtained a series of hybrid genes which had different proportions of lc to ompC formed by recombination between the PA2 and E.coli genomes. Their corresponding hybrid protein products were intermediate in properties between the protein 2 and the OmpC protein. With this they determined that the lc gene was in fact transcribed from right to left and is, therefore, coded by the opposite strand to the late phage genes. This explained why the lc gene is expressed in lysogens although located within the late gene region. At the same time, Gregg also determined that the

middle of the lc gene is located about 4Kb from the right end of the PA2 genome. These experiments confirmed that the lc gene is the structural gene for protein 2.

The proposal of Lee et al (1979) that phage PA2 acquired the lc gene by recombination with the E.coli chromosome gives rise to the question as to where this region could be. Defective prophages have been detected in the chromosome of E.coli K-12 strains. These are defined as λ-related DNA in bacterial strains classified as non-lysogens on the basis of the failure to elicit the formation of viable phage after treatment that would induce a λ prophage (Campbell and Botstein, 1983). The presence of such a prophage was first suggested by Low (1973) as a result of his investigation of the rac (recombination activation) locus, which was activated by zygotic induction in matings between recombination deficient K-12 strains. He proposed that the rac locus, situated a few minutes clockwise of the trp operon on the genetic map, is carried on a defective prophage whose functions are normally repressed but may be induced by transfer into a rac⁻ cell in which the prophage and hence the prophage repressor is absent.

The theory was supported by the observation of Gottesman et al (1974) and Gillen et al (1977) that λ reverse (λrev) phages (Zissler et al, 1971) are recombination proficient derivatives of λ in which the phage recombination functions have been replaced by analogous functions (RecE) derived from the host chromosome.

Kaiser and Murray (1979) confirmed Low's hypothesis by using restriction endonuclease analysis and DNA/DNA hybridisation methods.

They demonstrated that the Rac defective prophage is located at 31 minutes on the E.coli chromosome and has an estimated length of 27 Kb.

There is a group of mutant phages named λ<u>qsr</u>' (Q-independent) phages which are characterised by the replacement of the region of the λ genome which contains the <u>Q</u>, <u>S</u>, <u>R</u> genes with functionally analogous genes <u>q</u>, <u>s</u>, <u>r</u> derived from the E.coli K-12 chromosome (Fiandt <u>et</u> <u>al</u>, 1971; Henderson and Weil, 1975). The best studied are λ<u>p</u>4 (Jacob and Wollman, 1954), λ<u>qin</u>A3 (Court and Sato, 1969; Sato and Campbell, 1970) and λ<u>p</u>41 (Henderson and Weil, 1975). Southern hybridization showed that <u>qsr</u>' DNA is not carried by F'123 which carries the Rac prophage (Kaiser and Murray, 1979). Also, AB1157, a <u>rac</u>⁻ strain that cannot produce λ<u>rev</u>, lacks DNA homologous to the substitution in λ<u>rev</u>, but does contain other λ-homologous DNA corresponding to that found in C600 (Kaiser and Murray, 1979). From these results, Kaiser and Murray (1979) have proposed that there is a second defective prophage in E.coli K-12 bacteria, which is distinct from the Rac prophage. Restriction enzyme analysis and cross-hybridisation studies between cloned fragments of the <u>qsr</u>' defective prophage and λ DNA enabled Kaiser (1980) to map the approximate position of the <u>qsr</u> genes, of <u>cos</u> and of the <u>b</u>2 region. Fisher and Feiss (1980) also showed that in most of the K-12 strains <u>cos</u> and <u>Nu</u>1, accompanied by part of the <u>qsr</u>' substitution, can be rescued from the <u>qsr</u>' defective prophage by a mutant of λ, λ<u>cos</u>1, bearing a 400 bp deletion extending from <u>cos</u> into gene <u>Nu</u>1. Anilionis <u>et</u> <u>al</u> (1980) localized the <u>qsr</u>' defective prophage on F'152 by hybridizing a λ DNA probe with restriction fragments of a set of F

plasmid DNAs. F'152 carries a piece of E.coli DNA which starts from an IS3 element after purE and ends at a point after gal (Ohtsubo and Hsu, 1978). Therefore, the qsr' defective prophage should lie in a region between 12 and 17 min on the E.coli chromosome.

Recently, a third defective prophage in the E.coli K-12 chromosome was reported by Espion et al (1983). It carries genes analogous to Q, S, R of λ and also a cos site functionally identical to λcos. It is located near 34 min on the E.coli map.

The λ/PA2 heteroduplex (Figure 1.2) shows that the PA2 molecule has a piece of DNA which is non-homologous with the corresponding region in the λ molecule from 84.0% to 95.0%. On either side of this region, the PA2 and λ molecule are homologous. The lc gene of phage PA2 is located within this non-homologous region. In phage λp4, the λ DNA in this same region is replaced by foreign DNA. The DNA inserted in the λp4 molecule has been shown to be homologous to a region in the qsr' defective prophage which is also flanked by regions homologous to the λ DNA molecule (Kaiser, 1980). The λp4 molecule may have obtained the piece of DNA by homologous recombination with this defective prophage. Possibly the lc gene in PA2 has also come from this same prophage by the same means. Bearing this in mind, I made a heteroduplex with λp4 and PA2 DNA to look for homology in the lc region. The heteroduplex λp4/PA2 showed homology in the lc region as expected. In addition, it showed an insertion in the λp4 molecule relative to the PA2 molecule, possibly within the lc gene. This insertion may be related to the fact that λp4 lysogens produced no protein 2 and showed normal PA2 adsorption. I set out to answer following questions:

1) Does the insertion in the λp4 molecule exist in the qsr⁻ defective
prophage?

2) Does nmpC lie within the qsr⁻ defective prophage?

3) Is the insertion deleted in nmpC (p⁺) strains?

In the following Results section I first show that many of the
newly isolated phages described in the previous section appear to carry
the lc gene. I also show that a high proportion of all the phages
studied adsorb to protein OmpC.

From heteroduplexes made between PA2 and some newly isolated
phages (see Figure 2.2.), eight phages were found to be homologous
with PA2 in the lc region. The eight phages, 9, 15, 92, 93, 22, 28, 84A
and 84B are all temperate phages. They share homology with λ and are
UV inducible. It will be shown that four of these which were tested
all produce protein 2 as judged by SDS electrophoresis of the outer
membrane proteins or by reduced adsorption of phage PA2.

It was mentioned above that like PA2, phage 434 also uses protein
OmpC as its receptor. By analysing the heteroduplexes made between
various lambdoid phages (see Table 2.6), it was found that in gene J,
which produces the protein of the tail fibre by which the phage
adsorbs, phages PA2, 424, 84A, 84B, 92, 93 are all homologous. Phages
82, 434, 9 and 15 are also homologous with one another but differ
from the other six for about 10% near the middle of the gene
(segment 2).Phages λ and 21 however, differ from one another and
from all the others in the right 30% of the gene (segment 4). Since
PA2 and 434 adsorb to the same protein (OmpC) and λ adsorbs to a

different one (LamB) (Randall-Hazelbauer and Schwartz, 1973; Endermann et al, 1978), the specificity for adsorption must lie in this end 30%. I have confirmed this by measuring the adsorption rate of various phages to a mutant strain of K-12 which does not produce OmpC, and by spotting on this strain.

MATERIALS AND METHODS

Bacterial and bacteriophage strains

(See Table 3.1.)

Adsorption test

Bacteria used for adsorption tests were grown to an O.D. of 0.4 at
650 nm in 2mm cells, which was previously determined as the end of
exponential phase (Figure 3.1). Phages were added at a multiplicity of
0.1 PFU per cell (assuming 5×10^8 cells/ml), and the mixture was
incubated without shaking at $37^{\circ}C$. Samples were withdrawm at 1.5 to
10 min intervals depending on the adsorption rate of the different
phages to the control cells, and diluted $\times 10^4$ with L broth containing
200 ug of streptomycin per ml, which had been prewarmed to $37^{\circ}C$.
The dilutions were allowed to stand for about 10 min, and then they
were preadsorbed prior to plating by mixing the phage dilutions with
an equal volume of a seed culture of E.coli AB1157 in L broth. After
15 min at $37^{\circ}C$, 0.2 ml of this mixture was added to 2.5 ml of BBL
top agar at $50^{\circ}C$, and plated on BBL bottom agar containing 200 ug of
streptomycin per ml. Plates were incubated at $37^{\circ}C$ and plaques were
counted after 8 to 12 h.

Isolation and analysis of outer membrane proteins

Cultures in late exponential phase, the same stage of growth as
used for the adsorption test, were centrifuged at 4,000 rpm in a
benchtop centrfuge for 10 min at $4^{\circ}C$ and the pellets were
resuspended in 5 ml of buffer (10 mM Tris HCl, 5 mM EDTA and 2mM
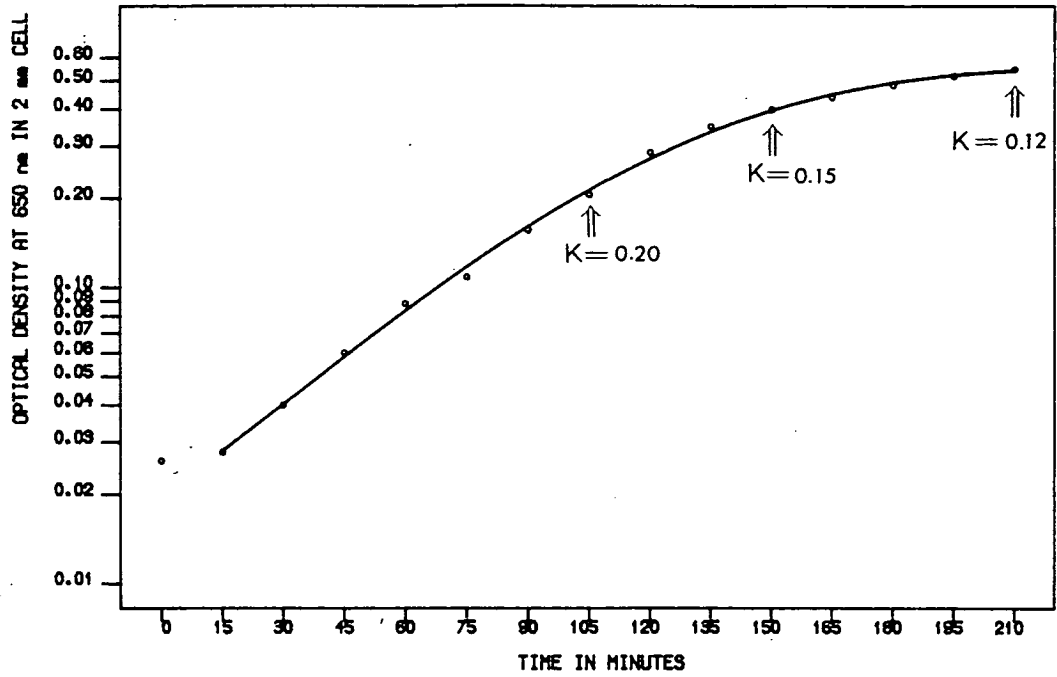
88

Figure 3.1 Growth curve of _E.coli_ K-12 strain C600 showing the variation in the adsorption constant for phage PA2.

Table 3.1a   Bacterial Strains

Strain	Relevant features	Source	Reference
C600	K-12	C.Ma	Appleyard(1954)
AB1157	K-12, str^r,	K.Begg	Low(1973)
C600(PA2)	Lysogenic for PA2	This work	
C600(λ)	Lysogenic for λ	This work	
C600(9)	Lysogenic for 9	This work	
C600(15)	Lysogenic for 15	This work	
C600(92)	Lysogenic for 92	This work	
CS419	EM3003, lysogenic for PA2ts9	C.Schnaitman	Pugsley et al(1978)
YC2	C600, ompC$^-$	This work	
C600(82')	Lysogenic for 82'	This work	
C600(434)	Lysogenic for 434	This work	
C600(424)	Lysogenic for 424	This work	
C600(λp4)	Lysogenic for λp4	This work	
C600[λqsr'(P$^+$)]	Lysogenic for λqsr'(P$^+$)	This work	
C600(λqsr')	Lysogenic for λqsr'	This work	
P1700	Wild type outer-membrane proteins	C.Schnaitman	Pugsley and Schnaitman (1978)
CS327	ompB156 of P1700	C.Schnaitman	Pugsley and Schnaitman (1978)
CS384	nmpC(P$^+$) of CS327	C.Schnaitman	Pugsley and Schnaitman (1978)

Table 3.1b   Bacteriophage strains

Strain	Relevant features	Source	Reference
λ	cI857Sam7	K.Murray	Goldberg and Howe (1969)
λclear		N.Murray	
λimm[434]		N.Murray	
NM1166	λatt⁻cI857supF	N.Murray	
NM698	λΔ(RI1-RI2)cI857 ninQam73Sam7	N.Murray	
NM22	λNam7Nam53	N.Murray	
λp4	b2imm[21]p4	I.Herskowitz	Fiandt et al(1971)
λqsr'(P⁺)	qsr' recombinant from CS384	This work	
λqsr'	qsr' recombinant from P1700	This work	
PA2	wild type	C.Schnaitman	Schnaitman et al (1975)
NM507	hλb2imm[21]clear	N.Murray	
NM848	h82imm[21]clear	N.Murray	

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mercaptoethanol, pH 7.8). The cells were then lysed by sonicating 3 times for 15 seconds (at a setting of 5 to 6 on an MSE 100 Watt Ultrasonic Disintegrator). The samples were cooled on ice for one minute between bursts. Unbroken cells were removed by centrifuging at 4,000 rpm for 5 min.

The supernatants were then centrifuged for 1 h at 20,000 xg. The pellets were resuspended in 0.5% Lauryl Sarkosyl by passing gently through a 23 gauge needle to dissolve the inner membrane. The outer membrane was then pelleted by centrifuging for 1 h at 20,000 xg. The pellets were resuspended again in 0.5% Lauryl Sarkosyl and centrifuged again for 1 h at 20,000 xg. The pellets were then resuspended in Tris buffer (10 mM Tris, pH 7.8).

Protein samples were mixed with an equal volume of x2 concentrated Laemmli buffer (Laemmli, 1970) and boiled for 20 min. The samples were then loaded on to a 7% to 20% SDS polyacrylamide gradient gel. Before mixing, each gel solution contained 0.375 M Tris HCl, 0.1% SDS, 13.6% sucrose, 0.027% Ammonium persulphate and 0.0275% TEMED in addition to containing 20% and 0.5% or 7% and 0.2% Acrylamide and bisacrylamide. Electrophoresis was carried out at room temperature overnight using a voltage of 100 V. Gels were stained with 0.1-0.5% Coomassie Blue (Weber and Osborn, 1969) for 3 h at 37°C and then destained in 45% methanol : 45% distilled water: 10% acetic acid.

Construction of supF bacterial strains

Phage NM1166 (λatt⁻cI857supF) was spotted on to L plates seeded with cells. The plates were incubated at 30°C to form lysogens. Lysogens were expected to form by recombination between the supF region of the phage and the E.coli chromosome. Cells from the centre ✗ of the spots were then streaked on to L plates spread with phage λ clear (10^9 pfu) and incubated at 30°C for 24h. The non-lysogens were expected to be killed by λclear. The best-looking surviving colonies were selected and streak out again. Individual colonies were then picked, grown up, and tested by spotting phages λ and λimm434 on them. The cells which were resistant to λ but sensitive to λimm434 were assumed to be lysogens.

The NM1166 lysogens were diluted and grown up again to avoid the contamination of λclear. They were then spread on L plates and grown at 42°C. Because they contained a temperature sensitive mutation in the cI gene most of the prophage were induced and lysed the cells. Only the cells which had lost the prophage were expected to grow at this temperature. Several hundred individual colonies were picked, and tested for the presence of supF by spotting on to plates spread with phage NM22 (λNam7Nam53) which requires supF for growth. Cells harbouring supF were lysed by NM22. Colonies which were indicated as harbouring supF were grown up and tested for lysis by phage NM698 (λQam73Sam7).

The formation of the qsr' recombinants

Three single plaques formed by NM698 [λΔ(RI1-RI2)cI857ninQ73S7] on $supF$ containing strains of both P1700 and CS384 were picked and grown to form plate lysates. (Since there was probably only a very small proportion of recombinants formed previously in the phage stock, and since all the phage in the NM698 stock could grow on the $supF$ strains the chance of picking one of such recombinants was low.) Phages from the plate lysates . (10^5 to 10^6 pfu) were then plated with the appropriate original strains of P1700 and CS384 which had no $supF$. Only gsr` recombinants were expected to form plaques. Individual plaques from each of the lysates were picked and propagated by forming plate and liquid lysates. DNA molecules of the phages were checked by forming heteroduplexes with the DNA molecules of λp4 and then observing them with the electron microscope.

RESULTS

Detection of the lc gene in some newly isolated phages

   a) Heteroduplex analysis of PA2 and some newly isolated phages

Heteroduplexes were made between the DNA molecules of PA2 and of some newly isolated phages. It was found that the DNA molecule of phage 15, 84B and 92 were homologous with that of PA2 in the lc region (Figures 2.2 j, 2.3 e, 2.2 h). According to Schnaitman (personal communication), the middle of the lc gene is located about 4Kb from the right end of the PA2 DNA molecule. This position can be marked at 91.3% on the PA2 genome if Kb is converted into percent of lambda. Heteroduplex PA2/15 (Figure 2.2 j) shows that from 84.8% to 96.2%, which certainly includes the lc gene, 15 is homologous to PA2. In the 92/PA2 heteroduplex (Figure 2.2 h), 92 is homologous with PA2 from 87.2% to the right end. The PA2/84B heteroduplex (Figure 2.3 e) shows that 84B is homologous with PA2 from 84.6% to 96.6%.

It was also found that in the lc region, the DNA molecules of phages 22 and 28 were homologous with that of 15 (Figure 2.3 g), the DNA molecule of phage 84A was homologous with that of 84B (Figure 2.3 b) and the DNA molecules of phages 9 and 93 were homologous with that of 92 (Figure 2.2 f, e). From that, we could predict that the DNA molecules of phages 9, 22, 28, 84A and 93 were also homologous with that of PA2 in the same region.

Thus, by heteroduplex analysis, I concluded that phages 9, 15, 22, 28, 84A, 84B, 92 and 93 all had the lc gene in their genome.

b) Adsorption tests for expression of the lc gene

First, to test the system, I made a PA2 lysogen with strain C600. The adsorption of phage PA2 to a culture of this lysogen and to a non-lysogenic culture of C600 was tested according to the conditions described by Schnaitman (1975). The test confirmed Schnaitman's result with strain W1485 namely that the rate of adsorption to the PA2 lysogen was significantly slower than the rate of adsorption to the non-lysogen (Figure 3.2). Lysogens of phages 9, 15, 84B and 92 were also made with strain C600. The PA2 adsorption test was carried out with these lysogens. Like the PA2 lysogen, they all showed reduced adsorption relative to the non-lysogen(Figure 3.2). I also showed that phage λ which does not contain the lc gene does not reduce the adsorption of phage PA2. Figure 3.3 shows that phage PA2 adsorbed to C600 (λ) and C600 at similar rates.

The adsorption of phage is generally represented by the equation:

$$\phi + B \xrightarrow{K} \phi B.$$

Where ϕ and B are the concentrations of free phages and bacteria respectively, ϕB is the concentration of bound phage and K is the adsorption constant (Gaven and Puck, 1951). When bacteria are in excess, the reaction can be presented by the equation:

$$\log \phi_0/\phi_t = KBt \text{ (Aebi } \underline{et} \ \underline{al}, \text{ 1973).}$$

Table 3.2 shows the relative K's determined from the adsorption tests. The ratio of the K of each lysogen to the K of the control culture
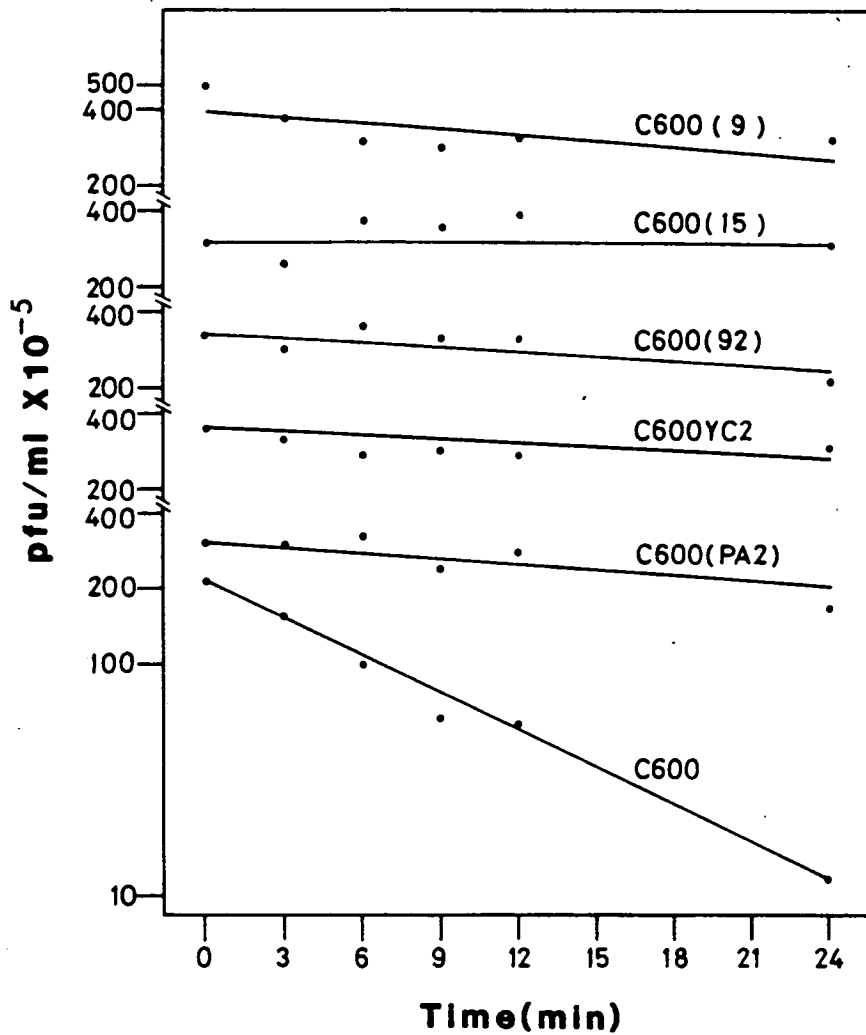
Figure 3.2 Reduction in the rate of adsorption of phage PA2 to strain C600 produced by prophages 9, 15, 92 and PA2 and by a mutation resulting in the absence of protein OmpC (strain YC2).
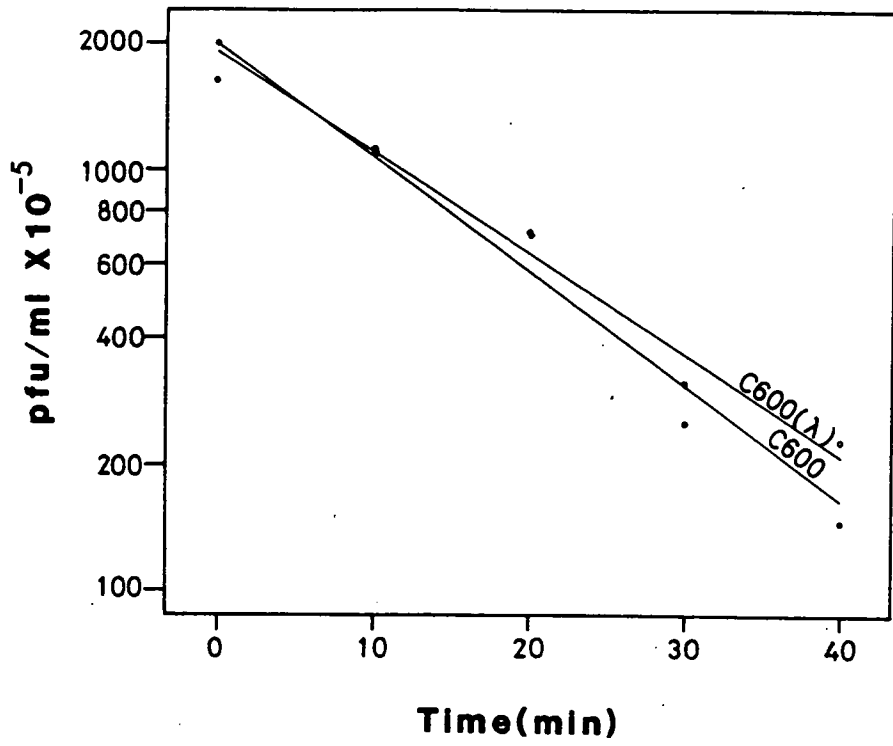
Figure 3.3 Control showing the lack of effect of prophage λ on the rate of adsorption of phage PA2.

Table 3.2   The Adsorption Rates of Phages to Bacteria Relative to C600[a]

Phage	PA2	9	15	92	λ	λp4	84B	YC1	YC2
			Lysogen of C600					Mutant of C600	
	(0.37)[b]	(0.23)	(0.05)	(0.14)	(1.37)	(0.74)	(0.03)	(-0.08)	(0.05)
	0.33	0.31	0.14	0.12	1.42	0.75	0.08	0.04	0.10
PA2	(0.23)	(0.13)	(-0.02)	(0.15)					(-0.10)
	0.15	0.16	0.01	0.14	0.89	0.78	0.03		0
	(0.13)							(-0.31)	(-0.07)
	0.23							0	0.02
82	(-0.11)								(0.12)
	0.02								0.06
									(-0.13)
									0
434c									(-0.08)
									0

a   The adsorption rate of phages to C600 is represented as 1.

b   The lower numbers are determined from the slopes of the lines in
    Figure 3.2, which were drawn by eye. The upper numbers, in brackets,
    are calculated by least squares analysis.

of C600 was calculated, so that the adsorption rates of PA2 to the different lysogens can be compared. In the adsorption tests, the O.D. of the control culture was made the same as that of the tested culture, for K depends on the stage of growth of the bacteria. This is shown in Figure 3.1. Three samples were taken at different O.D.s and tested for PA2 adsorption. K decreases as the cells approach stationary phase. The adsorption may also be sensitive to variations in the composition of the medium. When different batches of L broth were used, the adsorption rate of a given phage to a given bacterial strain varied, although the other experimental conditions were exactly the same.

c) Electrophoresis of outer membrane proteins

The outer membrane proteins of various strains harvested in late exponential phase, the same stage of growth as in the adsorption experiments above, were isolated and analysed by SDS polyacrylamide gel electrophoresis. Figure 3.4 shows that strains C600(λ), C600(82'), C600(434), and C600(424) all had the same bands as the non-lysogen C600. C600(PA2) showed an extra band (protein 2), as observed by Anthony and Schnaitman (1978) and the 1b band was much fainter than that of C600. Similarly, the lysogens harbouring phages 9, 15, and 92, which had the lc gene, all showed a protein 2 band and a reduced 1b band. The 1b band of C600(15) was hardly detectable.

By heteroduplex analysis, adsorption tests and electrophoresis analysis of outer membrane proteins, we have shown that the phages 9, 15, 92
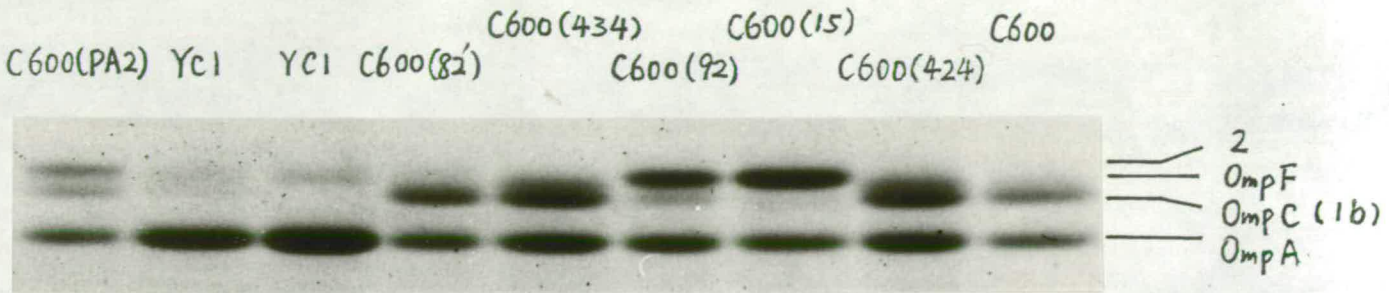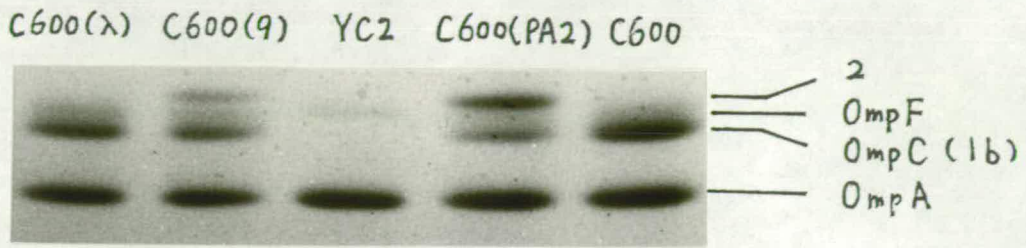
Figure 3.4 Polyacrylamide gel electrophoresis of outer membrane proteins showing the presence of protein 2 in lysogens of phages PA2, 9, 15 and 92 and the absence of protein OmpC in mutants YC1 and YC2. Cells all grown at 37°C. The numbering of the bands follows that of Bassford et al (1977).

and probably phages 84A, 84B and 93 have the ability to produce protein 2.

The phage gene defining adsorption specificity

By analysing all the heteroduplexes that we have made between 14 lambdoid phages, it was found that in segment 4, which is from 38.0% to 40.0% in λ and at the right end of the tail fibre gene J, there were 3 possible alleles (Table 2.6). Lambda has one, 21, 22 and 28 share a second and all the others have a third. We already know that the λ receptor is protein lamB (Randall-Hazelbauer and Schwartz, 1973; Endermann et al, 1978) and that PA2 and 434 both use OmpC as a receptor (Bassford et al, 1977; Hantke, 1978). Segment 2, just to the left, differs in 434 and PA2. Therefore segment 4 could be the part of the gene which determines the adsorption specificity of the phage as the remainder of gene J is homologous in all the phages. According to this, we can predict that any phage which is homologous with PA2 in segment 4, i.e. 424, 82, 9, 15, 84A, 84B, 92 or 93, uses OmpC as a receptor.

Two mutant strains of C600 were isolated which are resistant to 82 but not to λ or 21. The outer membrane proteins of these mutant strains, C600YC1 and C600YC2 were isolated and analysed by SDS-polyacrylamide gel electrophoresis. The result showed that protein OmpC (1b) was missing in these strains (Figure 3.4).

The adsorption test of PA2, 82, and 434 was done with C600YC2. None of these three phages showed significant adsorption to C600YC2 as

predicted (Figure 3.2, Table 3.2).

Spotting tests were done with the phages and C600YC1 and C600YC2. All the phages which have the same allele as PA2 in segment 4, as shown by electron microscopy heteroduplex analysis did not grow on these two strains (Table 3.3). Phages λ, 21, 22 and 28 did. Of a further 12 newly isolated phages which were tested, only 5 grew on C600YC1 or C600YC2, indicating that a total of 9 of the 26 phages tested did not adsorb to protein OmpC.

Shaw et al (1977) showed that host range mutants of λ were located in the C-terminal 10% of the J coding sequence, which is in the region of non-homology between λ and 434. Examination of the J protein peptide maps of these mutants showed that there were several peptides altered. Some of these altered peptides were derived from the C-terminal 10% of the J polypeptide. The J protein peptide map of 434 differed markedly from that of λ. These results are consistant with the observation that segment 4 of the genome determines the adsorption specificity of the phage.

The Ic gene in λp4

λp4 (Jacob and Wollman, 1954) is thought to be the product of recombination between λ and a defective lambdoid prophage (the qsr' prophage) (Herskowitz and Signer, 1974; Kaiser, 1980). About 11% of the lambda genome, between 84.0% and 95.0%, containing Q, S, R and the late gene promoter P'$_R$, is replaced by the p4 substitution, which is 20% λ units in length, coding for functions analogous to those deleted

Table 3.3   The relationship between _J_ gene alleles

              and adsorption to protein OmpC

              Alleles

              -------------------   Adsorption   Growth   Growth

Phages   Segment 2   Segment 4 to C600YC2   on C600YC1   on C600YC2

Phages	Segment 2	Segment 4	Adsorption to C600YC2	Growth on C600YC1	Growth on C600YC2
λ	λ	λ	n.d.	+	+
434	λ	434	-	-	-
82	λ	434	-	-	-
PA2	PA2	434	-	-	-
21	PA2	21	n.d.	+	+
424	PA2	434	?	-	-
9	λ	434	n.d.	-	-
15	λ	434	n.d.	-	-
84A	PA2	434	n.d.	-	-
84B	PA2	434	n.d.	-	-
92	PA2	434	n.d.	-	-
93	PA2	434	n.d.	-	-
22	21	21	n.d.	+	+
28	21	21	n.d.	+·	+

(Herskowitz, 1971; Fiandt et al, 1971). A heteroduplex was made between λp4 and PA2 DNA (Figure 3.5, Appendix 2.2). It showed that the PA2 DNA molecule was homologous with part of the p4 substitution and the lc gene was within this homologous region. Just about 1.5% λ units to the left of the centre of the lc gene, there was a loop. The size of this loop was about 2.7% λ units (Figure 3.5a). From the λ/p4 and λ/PA2 heteroduplexes, this loop was assigned to λp4 and indicates the presence of an extra length or insertion relative to the PA2 molecule.

Heteroduplex analysis indicated that the lc genes in λp4 and PA2 DNA molecules were homologous. In order to detect how much mismatch there might be in this gene, heteroduplex λp4/PA2 was spread in solutions containing different concentrations of formamide. The lc gene started to denature in 70% formamide. Among 6 heteroduplex molecules collected (Figure 3.5b), 3 molecules when measured were found to be denatured in the lc gene, the other 3 were undenatured in this region. Thus 70% formamide was just on the borderline of stability for this region. The homoduplexes, however, were not denatured until 75% formamide. So, the heteroduplex denatures in about 5% less formamide than the homoduplexes. According to the equations:

$0.65^{\circ}C = 1\%$ formamide, $1.4^{\circ}C = 1\%$ mismatch (see Section 1),

the mismatch between the lc genes of λp4 and PA2 is not more than 2 to 3%.

It was thus of interest to test whether the lc gene was expressed in a λp4 lysogen. However, because of the b2 deletion in the λp4 genome, half of the att site is missing. The λp4 genome does not
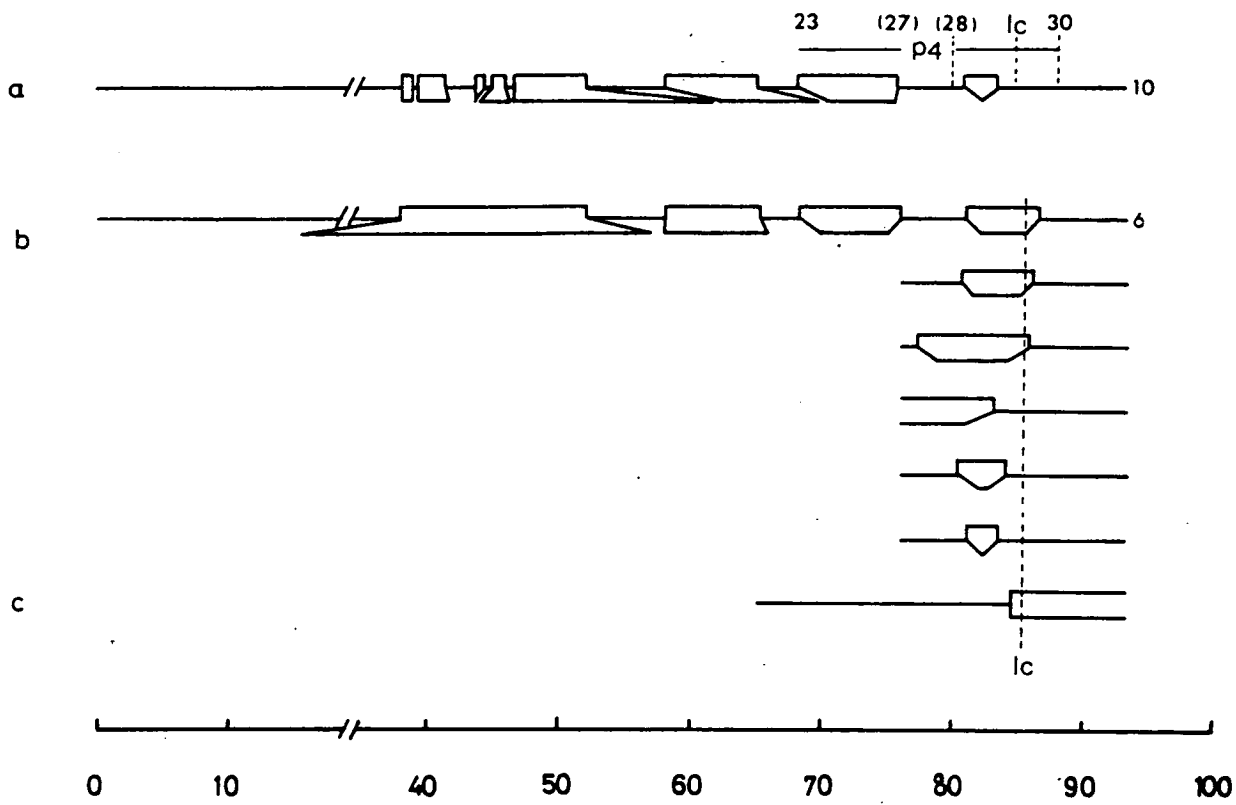
Figure 3.5 The λp4/PA2 heteroduplex a) hyperphase - 50% formamide b) hyperphase - 70% formamide c) homoduplex, right ends denatured in 75% formamide. (The number of molecules measured is given on the right; boundary positions are in Appendix 2.2; mode of assignment of single strands is in Appendix 2.3.)

insert into the E.coli chromosome at the normal λ att site. Considering the homology between the λp4 genome and part of the defective prophage in the E.coli genome, I thought that it might be possible to insert the λp4 genome by recombination into the p4 region. Thus C600 (λp4) lysogens were made under selecting pressure. First, phage λp4 was added to a C600 culture and allowed to have a short period of growth. Then, the culture was plated with phages λb2imm^{21}clear and 82imm^{21} clear, which do not form lysogens per se but destroy cells which are not λp4 lysogens or are resistant to λp4. The colonies surviving on the plate were picked up and tested by spotting on λp4 and 82imm^{21} clear. Ten double lysogens of C600 (λ+λp4) were also made in the same way.

These lysogens enabled me to do the PA2 adsorption test for lysogenic conversion. The result indicated that C600(λp4) and C600 adsorbed PA2 at approximately the same rate (Table 3.2).

The outer membrane proteins of nine individually isolated C600(λp4) lysogens and one C600(λ+λp4) double lysogen were extracted and analysed by SDS-polyacrylamide gradient gel electrophoresis (Figure 3.6). The protein composition of all of them was the same as for non-lysogenic C600. No protein 2 was detected.

From the evidence above, it can be concluded that there is an lc gene in the λp4 genome which is probably from the E.coli chromosome by a recombination event but which is not expressed in λp4 lysogens because of an unknown control mechanism.
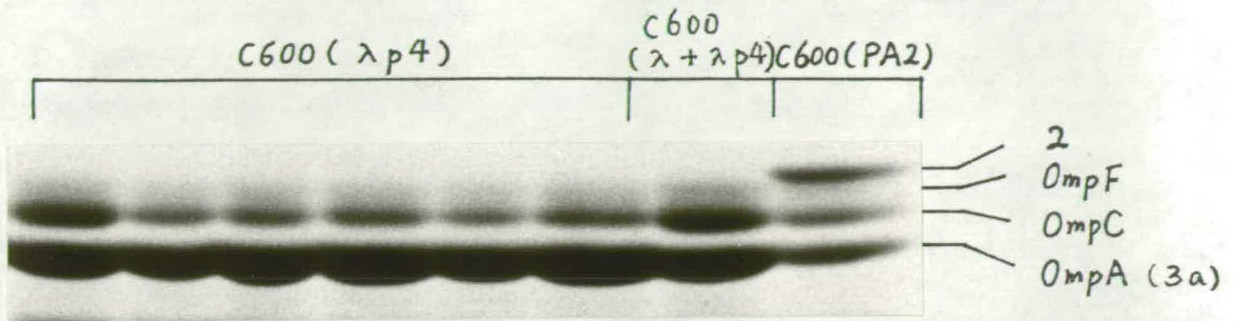
Figure 3.6 Polyacrylamide gel electrophoresis of outer membrane proteins showing the absence of protein 2 in 7 separate isolates of λp4 lysogens. Cells all grown at 37°C.

The location and control of nmpC

It has been shown that the DNA molecules of the phages PA2 and $\lambda p4$ are homologous in the lc region, apart from an insertion in $\lambda p4$, relative to PA2, just to the left of the middle of the lc gene, and that the lc gene was expressed in PA2 but not in $\lambda p4$. Therefore, the silence of the lc gene in $\lambda p4$ could be due to the insertion. Anilionis et al (1980) found that the qsr' prophage was carried by an F-prime factor (F'152). This factor carries a segment of the E.coli genome running from an IS3 insertion sequence after perE to a point after gal (Ohtsubo and Hsu, 1978). According to Pugsley and Schnaitman (1978b), gene nmpC is located at approximately 12 min on the E.coli map. This is very close to the qsr' prophage location. It seems likely that nmpC is in fact the lc gene carried by the qsr' prophage. Loss of the insertion could be the process by which the gene is activated in nmpC(P$^+$) strains. To test this I set out to generate λqsr' substitution mutants from an nmpC(P$^+$) strain CS384 and a wild type strain P1700 which was the progenitor of CS384.

a) Generation of λ phages with qsr' substitutions .

The phage used for generating substitution mutants was $\lambda\Delta$(RI1-RI2)cI857ninQ73S7. It has a deletion between the first and second EcoRI cleavage sites, which is from 44.7% to 60.2%, and also a nin deletion. The deletions were necessary to accommodate the extra length of the qsr' substitution (Strathern and Herskowitz, 1975). The phage also has an amber mutation in both Q and S genes. The

mutation in the $\underline{Q}$ gene can be suppressed by $\underline{supE}$ or $\underline{supF}$, and the one in the $\underline{S}$ gene can be suppressed by $\underline{supF}$. Since strains P1700 and CS384 did not contain $\underline{supE}$ or $\underline{supF}$, this phage was not able to produce viable particles on these two strains. The hybrid phages due to the recombination between the phage and the $\underline{qsr}^{\cdot}$ prophage $\underline{were}$ expected to grow. The frequency of recombinant formation was expected to be approximately 10^{-4} (Strathern and Herskowitz, 1975), which was the same as the expected frequency of reversion by point mutation of a single amber mutation in the $\underline{Q}$ or $\underline{S}$ genes. Since a phage bearing two different amber mutations was used, the frequency of reversion was reduced to 10^{-8}. So the recombinants could be selected by plating out the phage with the two strains and looking for plaques.

Considering there were likely to be recombinants in the phage stock already, it was necessary to prepare two new phage stocks which were grown up from single plaques on P1700 and CS384 respectively, so that recombinants could only be formed by recombination with these two hosts. In order to do that, it involved putting a $\underline{supF}$ gene into these two strains by allowing recombination to occur between the $\underline{supF}$ region of a transforming phage and the bacterial chromosome. This was done by making NM1166 ($\lambda\underline{att}^{-}\underline{cI857}\underline{supF}$) lysogens and then selecting for cells which subsequently lost the prophage but retained $\underline{supF}$. Since the phage NM1166 was $\underline{att}^{-}$, the integration of it was expected to occur by homologous recombination at the $\underline{supF}$ regions of the phage and the $\underline{E.coli}$ chromosome. Prophages were expected to be lost by the reverse process. Since excision need not always occur at the same point as integration a small proportion of prophages were expected to leave

their _supF_ gene behind.

Three recombinant phages were generated from each strain. The ones from P1700 were called λ_qsr'_; the ones from CS384 were called λ_qsr'_(P$^+$). As expected, heteroduplexes made between the DNA molecules of these two types of phages showed that they only differed by an insertion (Figure 3.7a). Heteroduplexes between the DNA molecules of λ_p_4 and those of the two phages were also made. The heteroduplex λ_p_4/λ_qsr'_ (Figure 3.7b) showed that the two molecules were homologous in the _p_4 region. The heteroduplex λ_p_4/λ_qsr'_(P$^+$) (Figure 3.7c) showed a deletion loop in the DNA molecule of λ_qsr'_(P$^+$) relative to the DNA molecule of λ_p_4.

b) The expression of the _lc_ gene in λ_qsr'_(P$^+$)

The next step was to see if the _lc_ gene in λ_qsr'_(P$^+$) could be expressed. Lysogens of C600 were made with phages λ_qsr'_(P$^+$) and λ_qsr'_. An SDS electrophoresis gel of the outer membrane proteins of the lysogens is shown in Figure 3.8. It revealed that the λ_qsr'_(P$^+$) lysogens produced an extra protein which had the same mobility as protein 2. This protein did not exist in the outer membrane of the λ_qsr'_ lysogens. However, the gel showed that the amount of the new protein was much less (relative to OmpF and OmpC) than that of protein 2 in the PA2 lysogen and unlike phage PA2, the lysogenization by λ_qsr'_(P$^+$) did not cause an apparent decrease of protein OmpF and OmpC. Also, the amount of OmpF protein, which is much less than OmpC in other lysogens and non-lysogens in normal growing conditions, is shown to be similar to that of OmpC in both λ_qsr'_ and λ_qsr'_(P$^+$)

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a   λqsr' : λqsr'(P⁺)

b   λp4 : λqsr'

c   λp4 : λqsr'(P⁺)

d   λp4 : λ
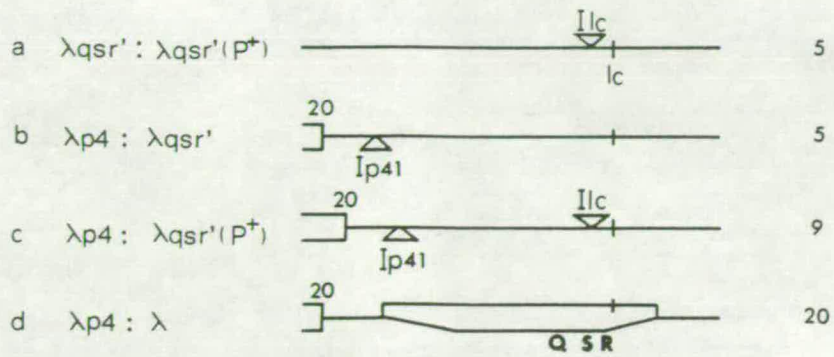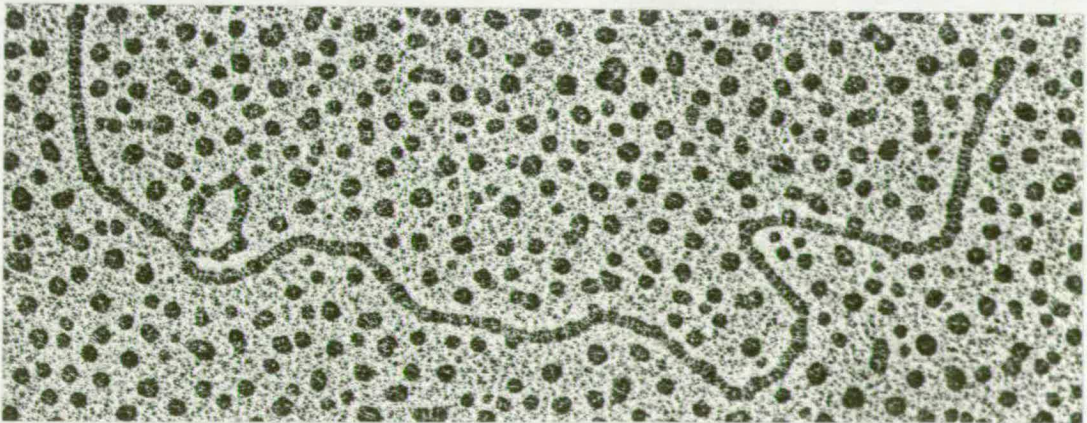
Figure 3.7 The right end of heteroduplexes between λp4, λqsr' and λqsr'(P⁺) DNA molecules showing the positions of insertions Ilc and Ip41. (The number of molecules measured is given on the right. The positions of the insertions and their mode of assignment is given in Appendix 3.1.)

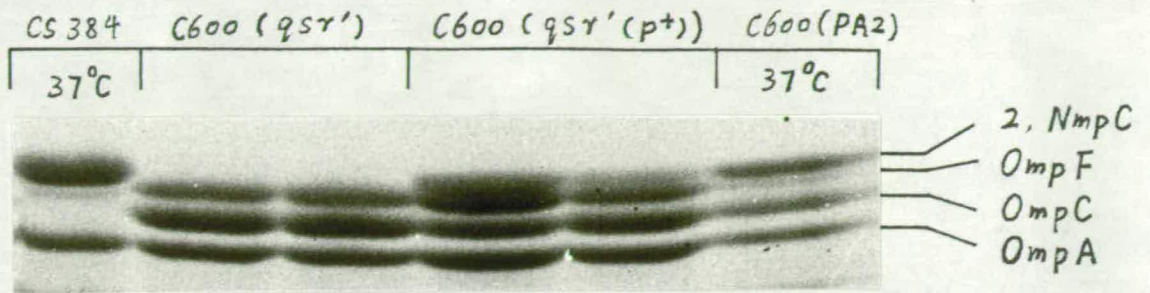The right end of the heteroduplex between the DNA molecules of λqsr' and λqsr'(P⁺).

Figure 3.8 Polyacrylamide gel electrophoresis of outer membrane proteins showing the presence of protein NmpC in lysogens of λ$\underline{qsr}$'(P[+]). [Two separate lysogens of λ$\underline{qsr}$' and of λ$\underline{qsr}$'(P[+]).] Cells grown at 30°C except where indicated.

lysogens.

The result of the PA2 adsorption test of the $\lambda \underline{gsr}'(P^+)$ lysogens is shown in Figure 3.9. No significant reduction of PA2 binding to the cell surface was detected.

What is the cause of these unexpected results? The protein composition of the cell outer membane depends on strain, growth phase, growth medium and growth temperature (Lugtenberg et al, 1976). The influence on the biosynthesis of the proteins OmpF and OmpC was found to be opposite: inductive for OmpF and suppressive for OmpC or vice versa (Lugtenberg et al, 1976; van Alphen and Lugtenberg, 1977; Koga-ban et al, 1983). The growth conditions of cells in my experiments were always the same except for the $\lambda \underline{gsr}'$ and $\lambda \underline{gsr}'(P^+)$ lysogens. Both phages were derived from phage NM698 which contains a temperature sensitive mutation in the cI gene. Therefore, the lysogens of these two phages were grown at $30^{o}C$, while the other lysogens harbouring wild type phages were all grown at $37^{o}C$. The observation by Lugtenberg et al (1976) that the ratio of the amounts of OmpF and OmpC increases at low temperature and decreases at high temperature is consistent with my results that the amount of OmpF is less than that of OmpC in cells grown at $37^{o}C$ but almost equals the amount of OmpC in cells grown at $30^{o}C$. The low production of the phage directed protein in $\lambda \underline{gsr}'(P^+)$ lysogens could also be related to the low temperature.

In order to show this, I regrew C600 and C600(PA2) at both $30^{o}C$ and $37^{o}C$. The SDS gel of the outer membrane proteins of the cells is
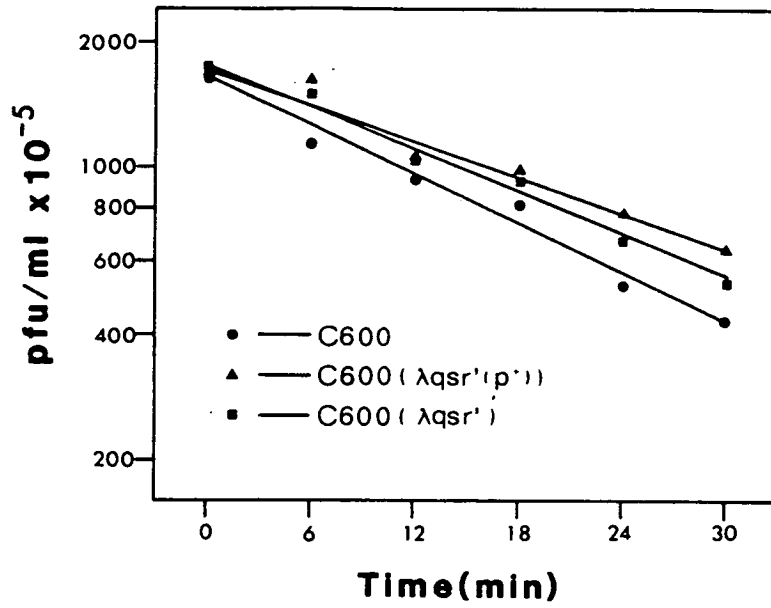
Figure 3.9 The similar rates of adsorption of phage PA2 to C600(λ*qsr*')
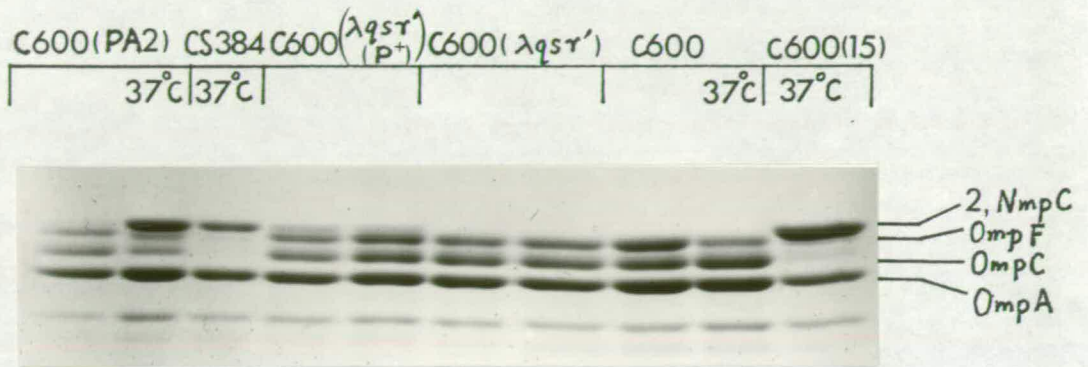and C600[λ*qsr*'(P⁺)].

Figure 3.10 Polyacrylamide gel electrophoresis of outer membrane proteins showing the reduced amount of protein 2 in PA2 lysogens grown at 30°C, and the increased ratio of protein OmpF to OmpC in C600 at 30°C. (Cells were grown at 30°C except where indicated.)

shown in Figure 3.10. The PA2 lysogen grown at 30°C showed the same composition as the $\lambda \underline{qsr}'(P^+)$ lysogens, ie, it produced much less protein 2 but more protein OmpF compared to that grown at 37°C. Also, as expected C600 grown at 30°C had more OmpF and less OmpC than at 37°C. So, we can conclude that the low production of the new protein in $\lambda \underline{qsr}'(P^+)$ lysogens, and the change of the OmpF to OmpC ratio detected in both $\lambda \underline{qsr}'(P^+)$ and $\lambda \underline{qsr}'$ lysogens, were due to growing at 30°C.

Subsequently I found that $\lambda \underline{cI}857$ lysogens can grow at higher temperature than 30°C. The $\lambda \underline{qsr}'$ and $\lambda \underline{qsr}'(P^+)$ lysogens were grown at 34°C and 36°C. The SDS gel of the outer membrane proteins of these lysogens is shown in Figure 3.11. The amount of protein NmpC in these $\lambda \underline{qsr}'(P^+)$ lysogens is much greater than in those grown at 30°C, and the ones grown at 36°C have more than those grown at 34°C. Also, these lysogens showed a lower OmpF to OmpC ratio at 34°C.

The DNA molecules of phages $\lambda \underline{qsr}'$ and $\lambda \underline{qsr}'(P^+)$ both contain an insertion to the left of the $\underline{p}4$ region, relative to $\lambda \underline{p}4$. It is shown as a small insertion loop in the heteroduplexes $\lambda \underline{p}4/\lambda \underline{qsr}'$ and $\lambda \underline{p}4/\lambda \underline{qsr}'(P^+)$ (Figures 3.7b and 3.7c). The insertion, which is 2.7% of λ in size, was named I$\underline{p}41$ since the location and size of it are very similar to a small piece of DNA in the $\underline{p}41$ substitution described by Henderson and Weil (1975). That piece of DNA was shown as a small loop just to the left of the $\underline{p}4$ substitution loop in the heteroduplex $\lambda \underline{b}221\underline{p}41/\lambda \underline{imm}434$. From its size it is possible that I$\underline{p}41$ is in fact a class I IS element.
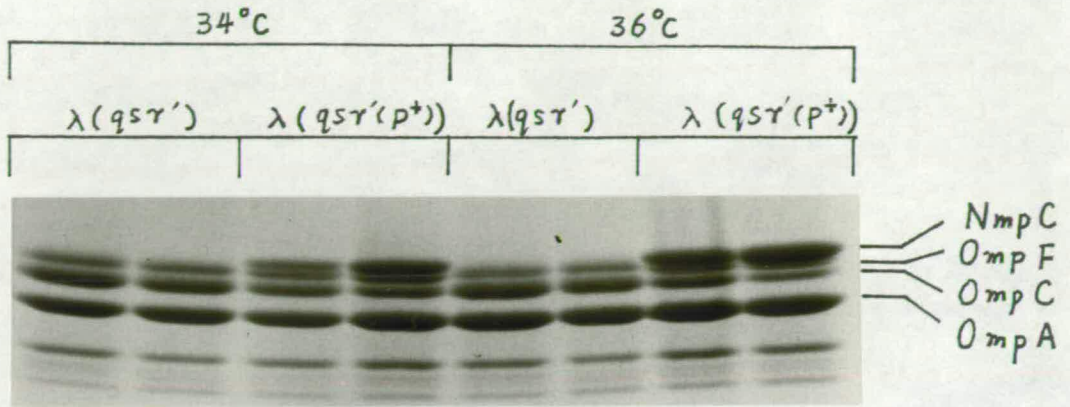
Figure 3.11 Polyacrylamide gel electrophoresis of outer membrane proteins showing the increased amount of protein NmpC in $\lambda \underline{qsr}'(P^+)$ lysogens grown at 34°C and 36°C (compare Figures 3.8 and 3.10). [Two separate lysogens of $\lambda \underline{qsr}'$ and $\lambda \underline{qsr}'(P^+)$.]

c) I$\underline{p}$41 in the $\underline{gsr}$' defective prophage

IS elements have been reported to effect recombination-related processes occurring in the λ genome. Phage $\lambda$$\underline{crq}$ (cryptogenic) is a variant of λ discovered by Fischer-Fantuzzi and Calef (1964), which is partially defective in $\underline{xis}$ function (Adhya and Campbell, 1970) and has the ability to produce cryptic prophage ($\underline{cry}$) at high frequency. A deletion covering the $\underline{int}$ through $\underline{R}$ region occurs most frequently in the cryptic prophage (Marchelli $\underline{et}$ $\underline{al}$, 1968). It was found by Zissler $\underline{et}$ $\underline{al}$ (1977) that $\lambda$$\underline{crq}$ has an IS2 insertion in orientation I at 60.7% , which is just upstream from the $\underline{xis}$ gene. They suggested that the insertion may exert a polar effect on $\underline{xis}$ and may also cause aberrant excisions leading to the formation of $\lambda$$\underline{cry}$ lysogens.

Friedman $\underline{et}$ $\underline{al}$ (1981) reported that a variant of phage λ, λaltSF, acquired the ability to substitute new sets of genes from the $\underline{E.coli}$ genome at high frequency owing to an IS1 inserted in the $\underline{cI}$ gene. However, there are some criticisms about this report (N. E. Murray, personal communication).

Another example of IS related recombination was reported by Fisher and Feiss (1980). The rescue of $\underline{cos}$[+] addition revertants seems to have involved two inverted repeat sequences, probably IS elements. The inverted repeats, one of which also exists in $\lambda$$\underline{p}$4, may be part of the $\underline{gsr}$' defective prophage, flanking the $\underline{gsr}$ genes and the λ-homologous DNA adjacent to $\underline{cos}$. The rescue of $\underline{cos}$ and $\underline{gsr}$ may have been performed by integration of $\lambda$$\underline{cos}$1 through homologous recombination into the $\underline{gsr}$' defective prophage via the λ-homologous DNA, and then

excision through illegitimate recombination conducted by the inverted repeat sequences (Fisher and Feiss, 1980). These sequences could be the same as IIc since one is within the p4 region and they are similar in size to IIc. The insertion Ip41 is just to the left of the p4 region, and might be responsible for the substitution of the p4 region from the gsr⁻ defective prophage in gsr⁺ recombinants. They could however, simply be formed by homologous recombination with the λ-homologous DNA known to exist on each side of the p4 region (Kaiser, 1980).

DISSCUSSION

In the outer membrane of cells lysogenic for phages PA2, 92 or 15, protein OmpC production was reduced significantly (Figure 3.4a). (It was not easy to detect the change in OmpF band since it was faint when cells were grown at 37°C.) The most apparent case was 15 lysogen, in which the protein OmpC was hardly detectable. However, in cells lysogenic for the phage 9 the amount of OmpC remained almost unchanged. Thus the reduction of OmpC in the outer membrane of these 4 lysogens appeared to vary over a wide range. It is not clear what caused this reduction, but by examining the SDS electrophoresis gel of the outer membrane proteins of these lysogens, it seems that the production of proteins OmpC and 2 are related. The more protein 2, the less protein OmpC. There might be competition between these two proteins in their production. The production of protein 2 could repress the production of protein OmpC. Alternatively, the competition could be in the process of secretion across the membrane and incorpration into the membrane. The differences in the amounts of protein 2 in the outer membrane of different lysogens could be due to slightly different structures of protein 2. Although the lc region in these lysogens all appeared homologous in the heteroduplexes there could still have been a small percentage of mismatch between them which was the result of point mutation. On the other hand, because of point mutations the promoters of the lc gene in different prophages may have different efficiency. The prophage with a very efficient lc promoter would produce a much larger amount of protein 2 compared with protein OmpC. Thus little OmpC would be incorporated into the outer membrane as in the lysogen C600(15).

By examining the SDS electrophoresis gel of the ten λp4 lysogens it appears that the protein OmpC may have been reduced in some strains relative to the non-lysogen (compare OmpC with 3a). The reduction of OmpC here is apparently not related to protein 2 since the lc is not expressed in λp4 lysogens. Phage λp4 had a b2 deletion extending from 45.3% to 57.3%, which contains the left component P of the att site (see Szybalski and Szybalski, 1979). Therefore, the integration of this phage into the E.coli chromosome does not occur at the normal λ attachment site, but at some other sites which share homology with λp4. The ten λp4 lysogens were made from a C600 culture grown from a single colony. The difference between them in protein OmpC production is possibly because the phage λp4 integrated into more than one site, and the integration at certain sites influenced the expression of the ompC gene. The double lysogen of λ and λp4 shows close to the normal amount of protein OmpC. The phage λp4 in this lysogen may have integrated at the normal λ integration site with the help of λ.

I have shown that eight of the newly isolated lambdoid phages which I have examined contain the lc gene. This suggests that phages having this gene are widely distributed in the Lothian Region. Schnaitman (1977) found that protein 2 was present in many wild strains of E.coli. However, none of five phages isolated earlier, namely, λ, 434, 82, 424, 21 contained the lc gene. Our phages were locally isolated, but Schnaitman's E.coli strains were from the east coast of America where the other five phages were isolated (Lederberg, 1951; Jacob and Wollman, 1956). Also, all five of these phages grew on the cells used in our isolation procedure, so phage such as these which lack lc were

not somehow selected against. Possibly lc was only recently transferred to phages by means of recombination. This is evidence for my speculation in the disscussion of Section 2 that phage genomes evolve rapidly through exchanging genes. However, phage DNA from a C600(21) lysogen recently obtained from the stocks of Dr. E. Wollman was homologous with the lc region of the phage 28 DNA molecule (see Section 2). The incorporation of the lc gene into bacteriophages may have biological significance. Most of the lambdoid phages studied use OmpC (1b) as a receptor. The replacement of OmpC by protein 2 prevents superinfection by phages which use OmpC as receptor but are heteroimmune.

It has been shown in this work that the nmpC gene in the E.coli K-12 genome is the same as the lc gene in phage genomes. The expression of this gene is controlled by an insertion both in bacteria and phages. From this, we can see that the evolution process of bacterial genomes and that of phage genomes are not separated events but are interdependent. They adopt genes from each other and apply them for their own benefit. The fact that the lc gene is on a defective prophage suggests that the defective prophage is not just genetic debris, it could play a role in the evolution of both phage and bacteria, through which, phages and hosts interchange diverged genes. When phages infect cells harbouring defective prophages some phages may recombine with defective prophages, producing new types of phages and providing the cell with new genes. Also, they can carry genes from one cell to another, so that bacteria can exchange genes between themselves. As a result, both phages and bacteria evolve.

Appendix 3.1 Assignment of loops in λ*qsr*′ heteroduplexes (Figure 3.7)

| Heteroduplex | I*lc* | | I*p*41 | | Boundary 20 |
	Position	Length	Position	Length	Position
a. λ*qsr*′/λ*qsr*′(P⁺)	10.2	2.1	-	-	-
b. λ*p*4/λ*qsr*′	-	-	26.5	2.3	30.5
c. λ*p*4/λ*qsr*′(P⁺)	10.2	3.0	24.5	3.0	28.6
d. λ*p*4/λ	-	-	-	-	30.3

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1. Positions are measured from the right end.

2. I*p*41 was assigned by comparing heteroduplexes b and d and I*lc* by comparing d and c.

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