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PROPHAGES AND GENETIC RESTRICTION IN *ESCHERICHIA COLI*, STRAIN W.

A thesis presented in in partial fulfilment of the requirements for the degree of Master of Science in Microbiology at Massey University.

Timothy Mark Doherty

1987

ABSTRACT.

E.coli strain W (ATCC 9637) is lysogenic for 2 phages which propagate on either E.coli C or E.coli K12. Each phage adsorbs specifically to only one of these hosts and the phages are therefore designated W_c and W_k respectively. These phages display a large number of similarities to phage Mu but restriction endonuclease digestion and probing with radioactively labelled DNA show that the W-phage are not identical to phage Mu.

Lysogens of E.coli C carrying either of the W-phage exhibit strong restriction towards phages P_1 and Lambda; lysogens of E.coli K12 carrying the same phage have not been shown to exhibit such restriction.

Conjugation experiments between E.coli W, E.coli C, and E.coli K12 allowed the mapping of the insertion sites of the 2 W-prophages on the E.coli W genome

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I would also like to thank all my friends in the Department who made the laboratory such an enjoyable place to work, and whose company also filled so much time, so well, outside the University.

T M Doherty.

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INTRODUCTION.

This thesis is primarily concerned with prophage-encoded genetic restriction systems expresed in E.coli strain W. Phage restriction is the expression of one of a number of systems which may prevent a phage from initiating a successful infection of a bacterial host cell. The term genetic restriction applies to the degradation by specific endonucleases, of DNA not native to the restricting cell. These restriction endonucleases recognise specific sequences of nucleotides in the DNA and cleave at, or near these sequences. This not only causes abortive infection by phage, but also restricts the transfer of cellular DNA by conjugation. The synthesis of restriction endonucleases may be directed by the host genome, by plasmids carried by the host, or by an endogenous prophage. Different strains may produce restriction endonucleases of differing activities.

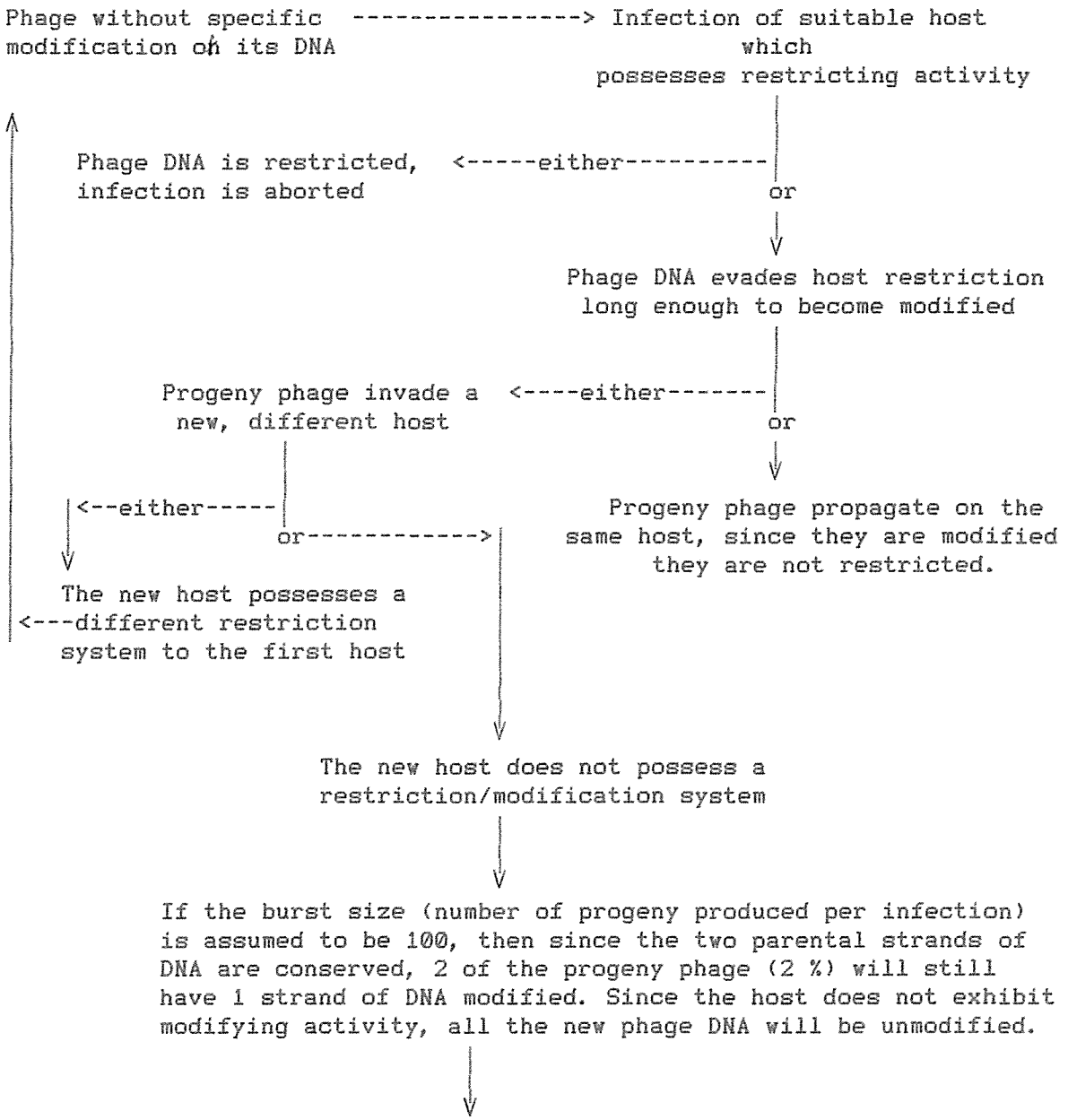
Restriction is almost invariably associated with modification. This may involve either methylation or glucosylation of the restriction enzyme recognition sites. Again, the genes coding for methylation may be located on the host genome, on a plasmid or a prophage. The modification system serves to protect the cell's own DNA from endonucleolytic attack, but will also protect the DNA of an invading phage that evades the effect of the restriction system.

Modification of DNA is not a genetic change, and is therefore not inheritable, although the genes which specify the enzymes responsible for modification obviously are. Phage which propagate on a modifying host retain this feature; each new generation of phage is modified as its DNA is replicated. However, a few generations of growth in a non-modifying

host will cause the modified DNA to be diluted out of the population [Arber (1965), Arber and Linn (1969), Hattman et al. (1966), Revel and Luria (1970)]; this can be best conveyed by the following diagram [see figure 1]

Genetic restriction involves the endonucleolytic scission of DNA; infection of a restricting host by a phage carrying DNA labelled with P^{32} [Dussoix and Arber (1962), Arber et al (1963), Smith and Pizer (1966), Glover et al. (1967)] has been shown to give rise to a number of breakdown products. The major DNA products of this process are acid-insoluble fragments, oligonucleotides, and inorganic phosphates (which are acid-soluble). Breakdown occurrs in two steps, first, relatively infrequent cuts along the length of the genome (dictated by the specific recognition sites) mediated by the restriction enzyme, then a slower degradation of the DNA by various, non-specific DNAases. The progress of restriction can be monitored by the increase in the proportion of label (P^{32}) which becomes acid-soluble with time. After a brief time, however, the restricting cell will begin to utilise the liberated phosphorus, so the level of label in the acid-soluble fraction will decrease.

The evidence for this two-step process in restriction includes the observation that there is some minimal expression of early phage functions in the restricting host, presumably before the DNA becomes totally degraded [Terzi (1968)]. Also, unmodified phage genetic markers can be retrieved in a restricting host by superinfection with a modified phage [Dussoix and Arber (1962)].



If the burst size (number of progeny produced per infection) is assumed to be 100, then since the two parental strands of DNA are conserved, 2 of the progeny phage (2 %) will still have 1 strand of DNA modified. Since the host does not exhibit modifying activity, all the new phage DNA will be unmodified.

Continued propagation in the non-modifying host. If 50 % of the progeny give rise to successful infections, 1 of these is likely to derive from the original modified parent. This new generation of phage will contain 50 x 100 new progeny, only 1 of which (0·02 %) is still modified. This "dilution" of the parent strand will continue until no modified DNA is left in the population. This is an ideal case of course; in reality, attrition due to inefficiencies in mating and breakdown of old DNA will remove modified parent DNA even more swiftly.

FIGURE 1 : DILUTION OF MODIFIED PARENTAL DNA OUT OF A POPULATION BY MAINTAINANCE IN A NON-RESTRICTING HOST

Above the molecular level, restriction is primarily visible as a decrease in the efficiency of plating of the restricted phage, or a decrease in the transmission of markers by conjugation. The level of restriction in any system depends on the physiological status of the restricting cells and also on the activity of the particular restriction endonuclease. Related to this latter factor is the nature of the of the genome which is being restricted. Some restriction enzymes respond to a recognition sequence of six nucleotides. These enzymes cut the DNA relatively infrequently, about once every 1024 nucleotides. Other restriction enzymes may recognise a sequence of four or five nucleotides. These enzymes, by simple random chance, will have a greater opportunity of binding to a recognition sequence and will therefore cut the DNA more frequently. Obviously, the greater the length of unmodified DNA exposed to the restriction enzyme, the greater the chance that recognition sequences will be available for the restriction enzyme to initiate an attack on the DNA. Arber has shown [Arber and Linn (1969)] that the loss of one recognition site from the genome of phage fd by mutation increases its efficiency of plating on the restricting host E.coli B from 7×10^{-4} (wild type, 2 recognition sites) to 3×10^{-2} (mutant, 1 recognition site). Statistically, the chances for a length of DNA, whether bacterial or phage in origin, with three or more recognition sites to survive exposure to restriction will be low. However such phages do propagate on restricting hosts (albeit at low frequencies). In these cases, survival is probably due to cells which are poor in their ability to restrict due to their physiological state. These weakly restricting cells still retain their modifying activity and the progeny phage may therefore be modified and able to initiate a spreading infection. Mutants totally lacking in

restrictive ability do exist, but they form a negligible part of the natural population and are less likely to provide centers of infection than the "weak" cells already mentioned. PB1395 (used in the work described this thesis) is an example of one of these restrictionless mutants which retains its ability to modify.

Various environmental factors such as the age of the culture, the composition of the media, and the length of incubation will influence the physiological state of the cells, and thus the efficiency of the restriction enzymes [Schell and Glover (1966), Arber and Linn (1969)]. If these factors are constant, the efficiency of plating of a given unmodified phage on a specific strain of bacteria is constant, and characteristic for that system.

In view of the relatively greater length of bacterial DNA, it is only reasonable to assume that there is a correspondingly greater number of potential restriction sites. This being so, conjugation between a restricting F^- (recipient) cell and an unmodified F^+ (donor) cell would seem unlikely to result in the transferral of much genetic information. However it has been observed by Glover, Copeland and other workers that approximately 20 minutes after mating commences the restriction system of the recipient appears to lose its effectiveness. It has been suggested that this is due to the saturation of the restricting endonucleases with unmodified DNA from the donor. The influence of restriction on bacterial mating has been thoroughly explored by Wood [Wood (1966)] and Arber [Arber and Morse (1964)].

Present evidence appears to suggest that the restriction endonucleases are located in the periplasm between the cell wall and the cell membrane. The evidence for this includes conversion of cells to spheroplasts

[Moholt and Frazer (1965)] and treatments which are known to remove cell membrane peripheral proteins, such as osmotic shock or treatment with EDTA [Schell and Glover (1966)]. Such treatment has been shown to decrease or eliminate restricting ability. Some workers [Schell and Glover (1966)] have infected restricting cells with unmodified phage at a temperature which decreased the efficiency of restriction. When the cells were resuspended in a hypertonic medium at 37°C restricting ability was restored, but a significantly increased number of phage were able to initiate sucessful infections, indicating that the restriction system was ineffective once the phage had penetrated the cell membrane. Control cells treated in the same way (barring pre-adsorption of phage at the lower temperature) showed no alteration in the efficiency of plating at 37°C when compared to untreated cells, so the increase in the number of sucessful infections was not due to damage to the restriction system of the treated cells.

These results are particularly germane to E.coli W, because the W-prophage-mediated restriction system does not appear to possess an associated modification system. It is the only modification-less restriction system covered in the literature. It is obvious that the genomes of both the phage and its host must be protected from digestion by their own enzymes by some other strategy. Furthermore, this method must be independant of any feature peculiar to E.coli W since the W-phage can successfully lysogenise other strains of E.coli. Strict compartmentalisation of the endonuclease in the periplasm of the cell is a likely method of protection. While it is possible that the W-phage might lack a recognition site for its own restriction enzyme, it is not plausible that the necessary site does not exist along the length of a

bacterial genome. In addition to this, the very marked effect that restriction has on bacterial conjugation observed in matings between sub-strains of E.coli W, indicates that the phage restriction is active against the E.coli W genome. Since there is reason to believe that a host factor is necessary for the activity of the phage-coded restriction system, the compartmentalisation of this hypothetical host factor could serve the same purpose as compartmentalisation of the enzyme and act as a further safeguard. E.coli W has its own chromosomally-encoded restriction and modification system of the hsd type but the modification by this system does not give protection from the W-phage mediated restriction endonuclease. The restriction systems operating in E.coli W are more fully described in the Historical Review and in Experimental section 1.

The restriction system of bacteria is a mechanism which protects the genetic "integrity" of the cell. It is ideally suited to its role as a first line of defence against phage, since its efficiency is greatest toward a phage which the cell has not previously encountered, a stage when neither cell or phage has evolved any mechanism of co-existance. Although restriction also inhibits the free exchange of genetic material, by conjugation, transduction or transformation and would therefore seem to be a limiting factor to the evolution of bacteria, some mechanism to preserve genetic isolation may not be a disadvantage.

The modification associated with most restriction systems has a protective role. DNA which survives restriction activity and is modified by the host is not restricted when it again enters cells of that strain. Since any restriction enzyme requires that both strands of a piece of DNA be unmodified before it can initiate an attack, the semi-conservative nature of DNA replication ensures that newly synthesised DNA will not be

restricted. The new DNA, being thus protected from restriction, will eventually become modified, and so will protect its own newly synthesised DNA when it in turn is replicated.

There are at least two different forms of modification. The first type involves specific methylation of either cytosine or adenine at the recognition site of the restriction enzyme, rendering that particular restriction endonuclease unable to bind to the DNA and cleave it. The crucial role of methionine in many modifying systems was suggested by the fact that modification was reduced in strains which were starved for methionine [Arber 1965]. Modification may also be reduced in cells which have been pre-infected with U.V.-irradiated phage T_3, which causes cleavage of s-adenosyl methionine, which is the substrate for the specific DNA methylase [Hirsch-kauffmann and Sauerbier 1968]. While these observations are strong indications of an important involvement of methionine in modification, they cannot be considered conclusive, since both involve gross alteration of the state of the modifying cells physiological state, and thus directly affect the growth of the phage.

A more direct link is provided by the determination of the relative degree of methylation of modified and unmodified DNA. It must be noted that most phage and bacterial DNA is non-specifically methylated. The relatively small proportion of extra methylations required to modify the specific recognition sites of the DNA is not easily distinguished. The DNA of phage fd however, is not heavily methylated. Using labelled 6-methyl-aminopurine (6-MAP) it was found that unmodified phage fd DNA has approximately 1 methylated adenine base per 4000 nucleotides. After modification by E.coli B the phage fd DNA carried 2 methylated adenine base per 4000 nucleotides. Since a single-stranded length of phage fd DNA

is about 6000 nucleotides, the B-specific modification added 2 methylated adenine bases. This agrees with the current evidence that unmodified phage fd DNA carries two B-specific sites on its DNA [Mamelak and Boyer 1970].

A second type of modification has been demonstrated with the T-even phage [Revel and Luria 1970]. These phage have hydroxy-methyl-deoxy-cytidylate (dHMP) instead of deoxycytidylate (dCMP) in their DNA. The hydroxymethyl group is glucosylated by glucosyl transferases using uridine diphosphoglucose (UDPG). This reaction is carried out by host enzymes induced and directed by the phage genome. If either the glucosyl transferases or UDPG are unavailable, modification of the phage DNA does not proceed, and the phage genome remains vulnerable to restriction.

There is a significant functional difference between these two mechanisms of modification, beyond the fact that they involve different enzymes and substrates. The bacterial modification system serves as a protective device against the cells own restriction enzymes should compartmentalisation prove insufficient. The bacterial modification system is therefore specifically intended to complement the bacterial restriction system.

The glucosylation of the T-even phage is a phage strategem unrelated to bacterial restriction/modification systems (except insofar as it is designed to circumvent them). It serves to preserve the DNA of the infecting phage from restriction.

Restriction-deficient (Res^-) mutants do occur in both bacterial and phage encoded systems [Arber and Linn 1969]. A significant number of Res^- mutants also lack the strain-specific modifying activity (Mod^-). With the exception of E.coli W, no Res^+ Mod^- mutants have been described in the

literature. A number of possibilities may account for this. It has been assumed that restriction without its concurrent modification is likely to prove fatal to the cell concerned. There is the possiblity that laboratory selection of mutants has focussed on changes in restrictive behaviour since there are no techniques available to directly select cells whose modification has been altered.

It has been shown that various strains of E.coli (E.coli K12, E.coli B, E.coli A, et al.) can lose all of their restrictive ability at a frequency compatible with a single mutation. This implies that there is a single locus carrying a gene for a single restricting endonuclease. The existence of this single locus has been confirmed by conjugation and transduction experiments. The locus has been designated the Host Specificity Restriction (hsdR) locus. Together with the genes for the DNA modifying (hsdM) and sequence-recognition (hsdS) functions, this gene forms a closely linked group located about 1 minute clockwise of threonine on the map of the E.coli chromosome in the different strains of restricting E.coli (E.coli K12, E.coli B, E.coli A, and E.coli W) [Arber and Linn 1969, Bachmann 1983]. These genes have been shown to be allellic by their ability to be transduced with the serine B or thymine R markers. The great similarity of these genes and their positions on the gene map suggest that the hsd genes of the restricting E.coli may be derived from a common ancestor.

The hsd locus includes 3 closely linked genes ; the hsdR, which codes for the restricting endonuclease activity, the hsdM, which codes for the modifying enzyme activity, and the hsdS, which codes for the protein which binds specifically to the recognition site that the preceding gene products act from.

Modification/ restriction systems can be divided into three groups on the basis of protein structure and their mode of action [Lewin 1983]. The simplest and most common (it occurs in about one in three bacterial strains) modification and restriction system is the type II. The type II restriction/ modification system has 2 genes coding for a restriction endonuclease and a modification methylase. Each of these enzymes, which is totally independant of the other, includes an active site which recognises the specific recognition site. The recognition site is often palindromic and is usually 5 - 6 nucleotides in length. The restriction endonuclease cuts at or near this recognition site, by breaking one bond in each strand of the DNA. Some enzymes may produce staggered cuts, giving rise to so-called "sticky" ends, others may make parallel cuts, giving rise to "blunt" ends. Because these enzymes are highly specific in the patterns in which they cut DNA, they are used extensively to examine the structure of DNA. EcoR1 is a good example of the basic type II restriction enzyme.

A subgroup of the type II restriction endonucleases, (possibly a step forward in evolutionary terms) are enzymes such as Hph, Mbo2, and Hga1, which cleave the DNA at a site slightly removed from the recognition site [Yuan 1981].

The generalised outline given above, of a 3-gene system, applies to the type I restriction/modification systems. This system is present in E.coli B and E.coli K12 (and the other restricting E.coli strains) and was the first restriction system explored at the genetic level. The association of three gene products was first suggested by complementation studies involving mutants of known phenotype. For example the diploid res$^-$ mod$^-$ / res$^-$ mod$^-$ was used as the recipient of an F-prime known to be

carrying *res*$^-$ *mod*$^-$ genes. Where complementation occurred, normal restriction and modification took place. It was shown [Arber and Linn 1969] that restriction and modification were restored simultaneously. This is most easily explained if the genotypes involved were *hsdR*$^-$ *hsdM*$^-$ *hsdS*$^+$ and *hsdR*$^+$ *hsdM*$^+$ *hsdS*$^-$.

The gene products are associated in a pentamer, with 2 restriction endonuclease , 2 modifying methylase , and a single site-recognition protein. Once the pentamer is bound to the DNA, either the restricting or the methylating activities can occur. These activities are mutually exclusive. Cells deficient in the *hsdM* or *hsdS* activities are *res*$^-$ *mod*$^-$; mutations in the *hsdR* gene are *res*$^-$ *mod*$^+$. There appears to be some mechanism to prevent a potentially lethal *res*$^+$ *mod*$^-$ phenotype. ATP is required for the type I enzyme complex to release from an already methylated recognition site or to modify or restrict an unmodified recognition site and it increases the efficiency of modification of the sister strand at a site which is hemi-methylated. The type I restriction enzymes cleave the DNA more than 1000 nucleotides from the recognition site. The exact mechanism is unknown, but electron-microscopic studies reveal looping of the DNA as it is being restricted, implying that the DNA is moved through the enzyme complex until a suitable site for cleavage is found. There is no information yet on what constitutes a suitable site for a type I restriction endonuclease to cleave, but it is not entirely random, since restriction occurs preferentially in some regions of DNA. Cleavage is a 2-step process. Initially, 1 strand is broken, then a second break is made in the other strand at a site nearby.

The type III restriction/modification system is coded for by 2 genes. One specifies a protein with *hsdR* activity, the other a protein with 2

domains carrying both hsdM and hsdS activities. The two proteins are associated in a dimer which requires ATP to bind to the DNA. Once bound, both restricting and modifying activities are expressed simultaneously, and each competes to complete its task before the other can. Modification takes place at the recognition site, while restriction takes place 24-26 base pairs away from the recognition site, presumably because of steric interactions. One interesting point in which type III systems differ from the other 2 types of restriction system is that the DNA is only hemimethylated by its modification system, that is, only the recognition site on one strand of the DNA is methylated. How modification is maintained during the replication of the DNA is unknown, but it has been suggested that the modification enzymes are linked to replication.

HISTORICAL REVIEW OF THE E.COLI STRAIN W SYSTEM.

Through the late 1940's and early 1950's considerable attention was focussed on elucidating the biosynthetic pathways of bacteria. The Waksman strain of Escherichia coli, (Escherichia coli strain W) was widely used as a standard organism for laboratory studies. It was deposited with the American Type Culture Collection and carries the designation American Type Culture Collection (ATCC) 9637. With the discovery of conjugation and P_1-mediated generalised transduction, increased attention was focused on the genetic basis for the cell's metabolic activities. However, when E.coli W was used as the host in transduction studies an interesting fact became apparent; E.coli W would not propagate the transducing phage P_1, although this phage could be shown to adsorb to the E.coli W with high efficiency [Glover and Aronovitch 1967]. The failure to propagate phage P_1 was shown to be due to the fact that E.coli W expresses very efficient restriction [Pizer et al. 1968]. This restriction is not only active against P_1, but also against phages T_1 - T_7 (with the exception of T_4, which does not readily adsorb to E.coli W), phage lambda and a number of other common coliphages. Several strains of E.coli (E.coli K12, E.coli B and others) exhibit restriction of coliphages, but none of these approach E.coli W in the efficiency of their restriction systems.

It proved possible to derive a mutant of phage lambda which would propagate on E.coli W [Glover and Kerszman 1967], presumably due to a mutation which altered a specific DNA recognition site for the E.coli W restriction endonuclease. In the course of this work, it was discovered that E.coli W was lysogenic for a previously undescribed temperate

bacteriophage, which was designated W$\bar{e}$. This phage was able to propagate on E.coli C, giving rise to turbid plaques 2-3 mm in diameter, very similar in appearance to those caused by phage lambda. Morphologically the W-phage resembles phages P_1 and P_2.

It was found by later workers [Pizer et al. 1968] that the W-phage coded for a restriction system which could be distinguished from the usual type I hsdR/hsdM/hsdS restriction system common to restricting E.coli. The inability of coliphages to propagate on E.coli strain W was explained by the fact that the prophage coded restriction system does not seem to have a concurrent modification system. Thus, any phage which evaded the restriction system and initiated a successful infection would still produce unmodified progeny phage which would be vulnerable to restriction on infecting another host cell. The possible mechanisms whereby E.coli W survives what has been speculated to be a lethal situation, that is, restriction without any protective modification, have been more fully discussed in the introductory section. To summarise that discussion, it seems likely that the E.coli genome escapes restriction since the restriction endonuclease is confined to the periplasm between the cell membrane and the outer cell wall. The overall efficiency of the restrictive ability of E.coli W is due to the presence in this strain of two restriction systems; a type I restriction system, apparently similar to the systems active in the other restricting E.coli strains. [Jamieson 1971, see also Experimental Section 1 of this thesis] and a restriction system coded for by the W-phage. Although it is likely that the first restriction/modification system noted is the same as the other type I restriction/modification systems found in various E.coli strains,

especially in view of the highly conserved nature of these other systems, it has not yet been conclusively demonstrated to be so.

Characterisation of the W-phages was a subject of the initial investigation in this laboratory [Jamieson 1971]. When other groups had investigated the phage released from E.coli W, they had propagated the phage on either E.coli C or E.coli K12, not both. When a chloroform-killed culture of E.coli W is titrated for the presence of phage, phage propagating on both E.coli C and E.coli K12 are found, with titres of approximately 10^6 and 10^5 plaque-forming units per millilitres (PFU/ml) respectively. Jamieson observed that these two different titres were the result of the presence of two different phage types in the suspension, rather than the result of a single phage with different efficiencies of plating on the two hosts. The two different prophages have been mapped to separate locations on the E.coli chromosome [Jamieson 1971, see also Experimental Sections 2 and 3 of this thesis]. One of these two phages can be shown to adsorb only to E.coli C, the other adsorbs only to E.coli K12. These phages were designated W_c and W_k respectively, since the specificity of each phage seems to be an inherited characterisic. E.coli K12 lysogens released phage which adsorb specifically to E.coli K12, while phage released from E.coli C lysogens adsorb specifically to E.coli C. Moreover, the adsorption coefficient of each phage to its specific host can be ascertained (the value of K is approximately 1×10^{-9}), while on the specific host of the other phage, the adsorption coefficient is too small to measure.

In addition, when the infective capability of each phage was measured against both hosts, a distinctive pattern began to emerge [see figure 2].

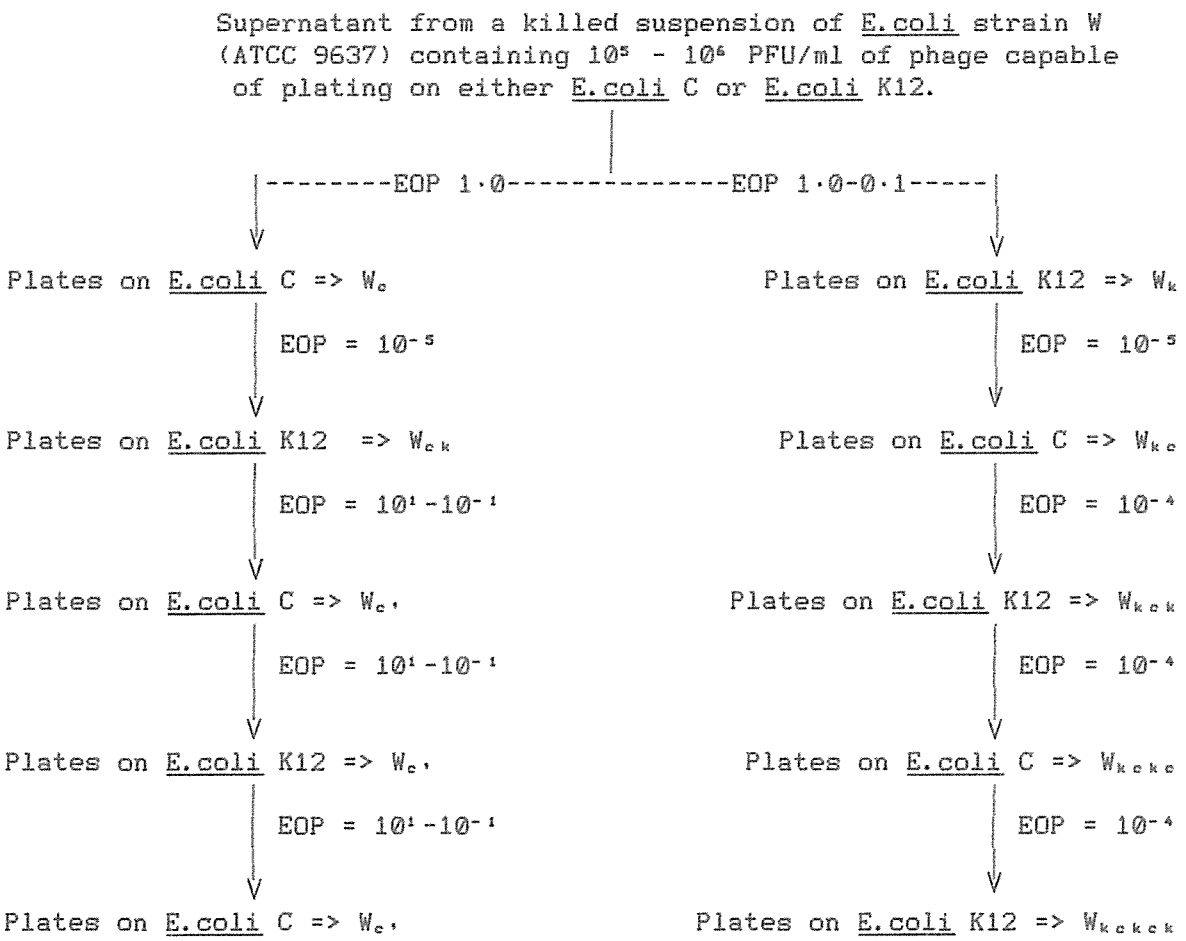


FIGURE 2 : PATTERNS OF PLATING OF Wk AND Wc ONTO E.COLI K12 AND E.COLI C.

If the supernatant from a killed culture of E.coli W is plated onto E.coli K12, it will give rise to plaques with an efficicency of plating defined as 1·0. If these phage are then propagated on E.coli K12, the EOP will remain at 1·0, but when these phage are propagated on E.coli C, the EOP drops to 10^{-5}. The phage which do plate on E.coli C are designated W_{kc}, and thereafter will propagate on E.coli C with an EOP of 1·0. However, when these W_{kc} type phage are plated back onto E.coli K12, the EOP falls to 10^{-5} again. This alternation of host range can be repeated indefinitely. This pattern is characteristic of systems which involve genetic "switching", in this case leading to alternating host specificities.

When the same experiment was attempted with phage W_c, however, a different pattern was observed. The initial plating of the W-phage from the E.coli W supernatant gave an EOP defined as 1·0 on E.coli C, and further generations of propagation on E.coli C maintained this EOP. When W_c grown up on E.coli C was plated onto E.coli K12 in an attempt to derive a W_{ck} type phage the EOP again fell to about 10^{-5}. To this extent the pattern of plating matches that of the W_k type phage. However, when these phage were plated back onto E.coli C, they did so with an EOP equal to that on E.coli K12, and this EOP could be demonstrated thereafter on whichever strain of E.coli (K12 or C) was chosen as a host; this extension of host range remained stable and inheritable. This pattern of behaviour is characteristic of an extension of host range [Jamieson 1971]. Similar host-range mutations were also observed by other workers [Glover et al. 1967]. These workers also came to the conclusion that this was the result of a mutation which led to an extension of host range. The host-range mutant of W_c was designated W_c'. It was suggested [Rice 1980]

that the occurrence of the host-range mutant which arises with a frequency of 10^{-4} to 10^{-5}, masks an alternation of host range analogous to the behaviour of W_k. This would be true if the host range mutant arises with a greater frequency than does genetic switching.

The work on alternation of hosts and host-range extension was continued by later workers [Rice 1980]. The relative proportions of the results obtained in this work were approximately the same as those reported earlier [Jamieson 1971], however both the EOP and the overall phage titres were significantly decreased. The causes of this have not been established. Other workers [Van de Putte et al. 1980] have commented on the difficulty of maintaining stable titres with phage Mu and Martha Howe [Personal communication] has attributed this to variations in the levels of divalent ions in the water. While titres, of phage Mu in particular, can still show a major decline after a period of storage, this problem has been somewhat alleviated by the use of Milli-Q water and careful monitoring of the levels of divalent ions in media and especially in buffers. Concentrations of Ca^{2+} and Mg^{2+} have proven to be most important in this regard.

The work done in this laboratory on the identification of phage specificity with specific anti-sera also provided useful results. It proved possible to raise neutralising anti-sera in animals to the W_c and W_k phage. Anti-sera raised against W_c will not neutralise W_k and vice versa. However, when W_k undergoes conversion to the W_{kc}, propagating on E.coli C, it is no longer neutralised by W_k anti-sera but instead is neutralised by the anti-W_c anti-sera. The W_c' extended host-range type, however, behaves as predicted for a mutation of this type; despite being able to to propagate on E.coli K12, the W_c' phage is not neutralised by

anti-W_k anti-sera, but is by anti-W_c anti-sera. This implies that there has been some change in the existing W_c attachment proteins of the phage, rather than a change to entirely new W_k attachment proteins, with a concurrent change in host specificity. The results obtained with W_k, imply a completely different state of affairs. Even over a large number of alternations, from the W_k-type to the W_{kc}-type to the W_{kck}-type, and so on, the phage is always neutralised by the anti-sera specific to the type of phage which would normally propagate on that host.

Extended host range mutants of W_k (W_k') have been isolated, albeit with a lower frequency, but only from phage which have been propagated previously on E.coli C (that is, only W_{kc}' or W_{kckc}' rather than a true W_k'). It would appear that a mutation causing extension of the host range of the W-phage is only expressed when the genome is in the arrangement which allows propagation of the phage on E.coli C [Rice 1980]. Extended host range mutants derived from W_k or W_c are both neutralised by anti-sera raised against the W_c tail form.

Although the two W-phage display some differences in behaviour, there are a large number of similarities. W_c and W_k are co-immune, they display sensitivity to heat that is identical over a wide range of ionic concentrations (within the limits of error) and anti-sera raised against phage W_{kc} neutralises W_{ckc} and vice-versa. The neutralisation coefficients have been shown to be the same in either case. Neither of the W-phages is induced by ultra-violet light unlike many other temperate phage, such as phage lambda. Both W_k-type and W_c-type phage are identical in size and morphology, and are similar in these attributes to phage Mu, which shares a number of these other characteristics as well (see figure

3). The W-phage possess spherical heads, about 65 nanometres (nm) in diameter. The phage tail is about 180 nm long, and 16 nm in diameter. When contracted, the sheath is 26 nm in diameter, and the exposed core of the tail is 8 nm in diameter. At high concentrations, these phage appear clumped together in electronmicrographs, tails touching to form the so-called "rosette formation" [Jamieson 1971]. In the opinion of M. Howe, [Personal communication] this is most likely to be an artifact of preparation caused by attachment to nutrients and other debris in the medium.

The position of the W-phages on the E.coli W chromosome was tentatively mapped out by Alan Jamieson [Jamieson 1971]. That work has been carried further in this thesis. Evidence was obtained by Jamieson that there are at least two distinct prophages which code for the W_o and W_k phage. If genetic switching is occurring at all in the prophages of E.coli W, it is at a very low level.

Comparison of the genetics of phage Mu with the W-phage is both valid and useful, since the two phage do appear to be closely related. In 1963 it was reported [Taylor 1963] that a previously unknown temperate phage had been discovered. It was found by chance when a culture which had been exposed to P_1, was examined for the presence of lysogens of P_1. A bacteriophage was isolated which gave rise to plaques which could be distinguished from those of phage P_1 and which, when examined, proved to be different morphologically from P_1. The most interesting feature of the new phage was its ability to induce mutations by prophage insertion within a stretch of functional DNA, rendering that gene nonfunctional; this insertion was essentially random. The new phage was designated Mu-1 for Mutator phage.

SIMILARITIES BETWEEN PHAGE Wk AND PHAGE Mu.

- Both are temperate, non-inducible phages
- Both propagate lytically on E.coli K12
- Both phages, when propagated lytically on E.coli K12, fail to propagate on C.freundii but induced lysates of each phage include some phage with an altered host range which do plate on C.freundii
- Both have similar morphology under E.M.
- These phages are co-immune
- Plaque morphology of both phages are similar
- Both phage induce insertion mutations by random insertion of the prophage
- Antisera to phage W_k gives full neutralisation of phage Mu_k

DIFFERENCES.

- Mu plates on species of Enterobacteriaceae that the W-phage will not plate on
- A alternate host specific phage of the Mu_{kc} type (comparable to the W_{kc} type) has not been observed
- W_k phage are associated with restriction, Mu phage is not

FIGURE 3 : COMPARISON OF CHARACTERISTIC PROPERTIES OF PHAGE Mu AND PHAGE Wk.

In a later paper Taylor described [Martuscelli et al. 1971] a source of the Mu phage used in their paper as follows;

> "The mutagenic phage used in this investigation was isolated from the American Type Culture Collection (ATCC) strain 9637, by plating cell free culture medium with indicator cells, E.coli K12 (strain AB 259). Since the phage from ATCC 9637 is indistinguishable from the phage Mu-1 orignally isolated from a chance lysogen of strain K12, in respect to its morphology, lysogenic immunity, bacterial host range, and ability to induce mutations, we presume that the two phage are closely related, and that ATCC 9637 may be the natural reservoir of phage Mu-1."

This is, of course, exactly the method by which the W_k phage has been isolated by workers in this laboratory [Jamieson 1971, Rice 1980]. With the exception that the restrictionless E.coli K12 mutant, PB 1395, is now used, this is still the method by which initial isolations of the W_k phage are made.

There are a few additional notes to be appended to the statements made in this quote in the light of more recent research. Mu is now known to plate on Shigella sonnei, Serratia marcescens, Enterobacter cloacae and other strains [Bukhari and Ambrosio 1978]. This is not a feature shared by the W-phage, either those with the normal host specificities, or the extended host-range mutants. In addition, the W_c phage (which of course was not detected by the workers previously alluded to since they isolated their phage by propagation on E.coli K12), only propagates on, and adsorbs to, E.coli C. While phage Mu will propagate on E.coli C, this appears to be due to a Mu_k, host range mutant. The pattern of propagation of phage Mu propagated initially on E.coli K12 (Mu_k) is notably different

from that of W-phage propagated on E.coli K12 (W_k), if both are then plated onto E.coli C. W_k will plate on E.coli C (giving rise to a $W_{k\,c}$ type phage) with an EOP of about 10^{-5}, while Mu_k propagates on E.coli C (giving rise to $Mu_{k'}$ host range mutant type phage) with an EOP reported to range from 10^{-9} [Van de Putte 1980] to 10^{-11} [Moody 1983].

Finally, the two W-phages have been associated with a phage-carried restriction system [Smith and Pizer 1966, Glover and Kerszman 1967, Pizer et al. 1968, Jamieson 1971, see also this thesis, Experimental Section 1]. In contrast, phage Mu has not, to our knowledge , provided any evidence that it carries genes involved in restriction. This is despite a stringent examination of various strains of E.coli made lysogenic for phage Mu [Rice 1980, Moody 1983]. These differences are certainly significant, and warrant attention , notwithstanding the large number of apparent similarities between the W-phage and phage Mu. While the evidence for some close relationship between the two phage is good, there is enough evidence to suspect that they may not be identical.

In addition to the link between W_k and Mu drawn by Martuscelli and Taylor, and the similarities discussed above, (see also figure 3), the most intriguing case in which parallels can be drawn between the W-phage and phage Mu is their behaviour when propagated on E.coli K12 and Citrobacter freundii. The alternation of host range displayed by the W_k phage on E.coli K12 and C.freundii is similar to behaviour reported for phage Mu [Bukhari and Ambrosio 1978, Van de Putte 1980]. These points of similarity, in particular this last item, led to a study directly comparing phage Mu to the W_k phage (and to a lesser extent, the W_c phage). The final conclusion drawn from this study was that the two phage are closely related, if not identical [Rice 1980]. Unfortunately, this

work was not able to conclusively address the question of exactly how close this relationship is, and whether the phage are, in fact, the same.

Mu has proved to be a fruitful field for research, and has been extensively studied [Taylor 1963, To 1968, Abelson et al. 1973, Bukhari et al. 1977]. Morphologically, the Mu phage is similar to the W-phage and also to phage P_1. The phage is composed of an icosohedral head about 54 nanometres (nm) in diameter, with a contractile tail sheath some 100 nm long and 18 nm in diameter when not contracted. The tail is capped with a base plate and at least 3 tail spikes [To 1966, Matuscelli et al. 1971]. The genome of Mu is linear, double-stranded DNA, about 36 to 38 kilobases (kb) in length. This corresponds to a molecular weight of about 25 megadaltons (Mdal) [Daniell et al. 1973, Bukhari et al. 1977]. The phage genome has several unusual features in addition to its ability to induce mutations by random prophage insertion into functional genes of the host. These features include the presence of fragments of host DNA at the ends of the phage genome and the possession of a gene-splicing mechanism associated with the inversion of a segment of DNA (the G-region); this DNA inversion and the associated gene splicing is responsible for the "switching" of host specificity in phage Mu [Van de Putte et al 1980].

The presence of host DNA at the ends of the Mu genome is a result of the method of packaging employed by this phage, a method which ensures that all of the phage genome is transfered into the phage head by cutting the host DNA beyond the ends of the phage genome. The method of packaging the genetic material into the phage head is by a process referred to as "headful packaging" [Chow and Bukhari 1977]. Although the fragments of host DNA are variable since they may be from any of the several random insertion sites within the host genome, they are of relatively constant

size as this is controlled by the phage's replication system. At one end of the phage Mu genome (the "c" end) the host DNA sequences are approximately 0·1 kb in length and are barely visible in heteroduplex complexes under the electron microscope. At the other end of the Mu genome, (the "s" end), the host DNA sequences are approximately 1·5 kb in length in wild type phage Mu. This terminal host DNA is easily visualised in electronmicrographs as a "split end" in heteroduplex studies. They may also cause a blurring effect on bands of restriction fragments less than 10 kb long which includes these variable regions under agarose gel electrophoresis. The Mu genome is, by convention, represented in diagrams with the "c" end on the left and the "s" end on the right .(See figure 4)

The G-region, the invertable segment of DNA controlling the adsorption of the phage to its various hosts [Daniell et al. 1977] is a 2·9 kb length of DNA (+/- 0·4 kb) flanked by a pair of inverted repeat sequences 50 base pairs (bp) in length. The G-region is located approximately 1·6 kb from the "s" end of the Mu phage DNA, or approximately 2·1 kb from the end of the complete free virion genome (including the variable host sequence). The G-region and the adjacent DNA codes for the S and U gene products, which are tail proteins involved inrecognition of attatchment sites on the surface of the specific host cell [Howe et al 1979, Giphant-Gassler et al 1981]. Inversion of the G-region, which contains two different terminal sections for a functional S gene, splices these "part genes" with the constant section of the gene, on the phage genome outside the invertable region. This produces proteins with a constant domain (coded for by genes outside the G-region) and a variable domain (coded for by genes inside the G-region) [Bukhari et al. 1977]. See figure 5.

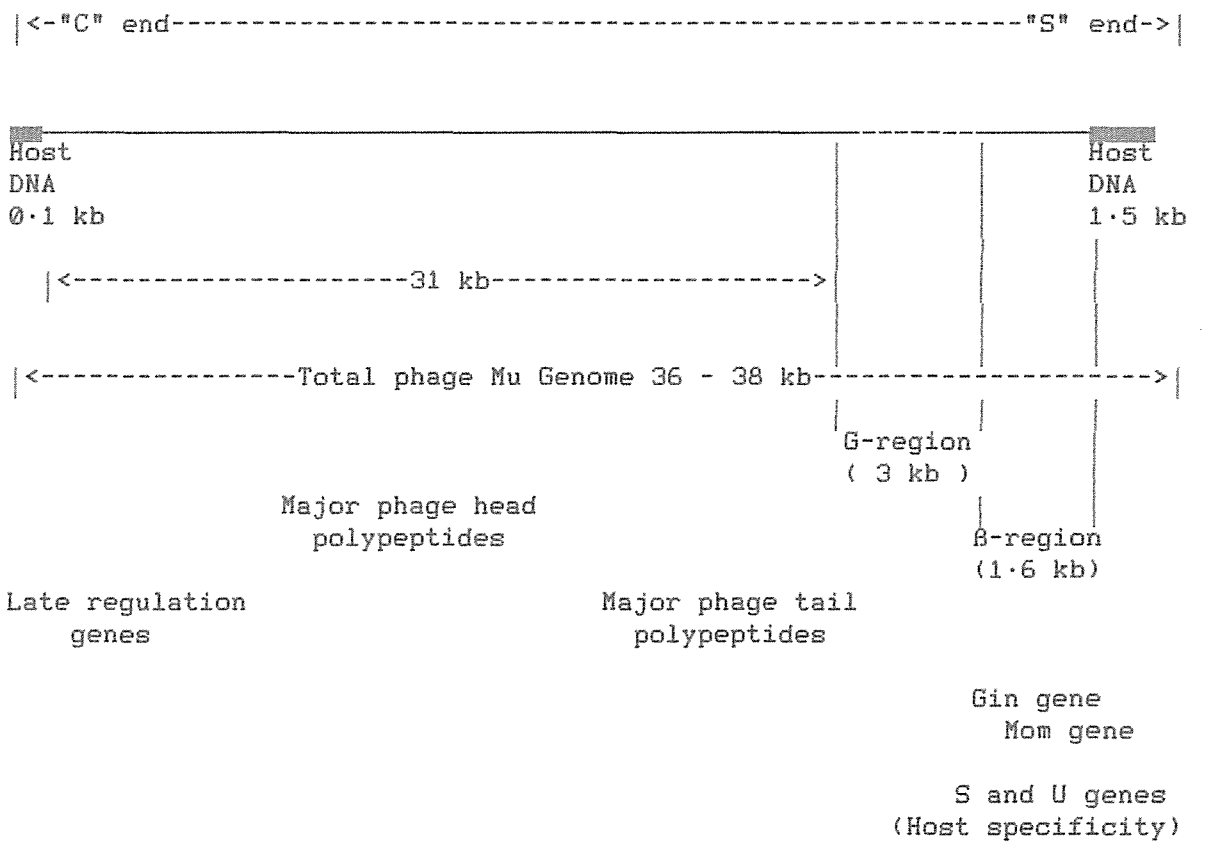


FIGURE 4 : STRUCTURE OF THE GENOME OF PHAGE Mu.

The fact of greatest interest from the point of view of a study of the W-phage, is that the G-region in phage Mu is homologous to the C-region in phage P_1, which controls the specificity of that phage. There are minor differences between the C-region and the G-region, and the C-region has 650 bp flanking inverted repeats rather than the 50 bp found in the G-region of Mu. We have considered the possibility that a similar gene-splicing mechanism may account for the alternation of host range displayed by the W_k phage, and this problem has been addressed in Experimental Section 4 of this thesis.

Inversion is thought to occur by recombination between the inverted repeats, a process which is mediated by the G-inversion (Gin) gene in phage Mu, or the C-inversion (Cin) gene in phage P_1. In both cases, these inversion-mediating genes are linked to the invertable region of DNA, although the gene product has been shown to be trans-acting; the G-region of Gin^- mutants of phage Mu is inverted when a lysogen carrying this mutant phage is superinfected by a Gin^+ helper phage.

The G-region in Mu has another novel feature; inside the invertable segment is another region flanked by invert repeats which, in theory might be capable of inverting. This region is quite small, about 1 kb in length,including the inverted repeats. There is no evidence as yet, however, that this small segment can invert, or that any inversion which may occur has an appreciable effect.

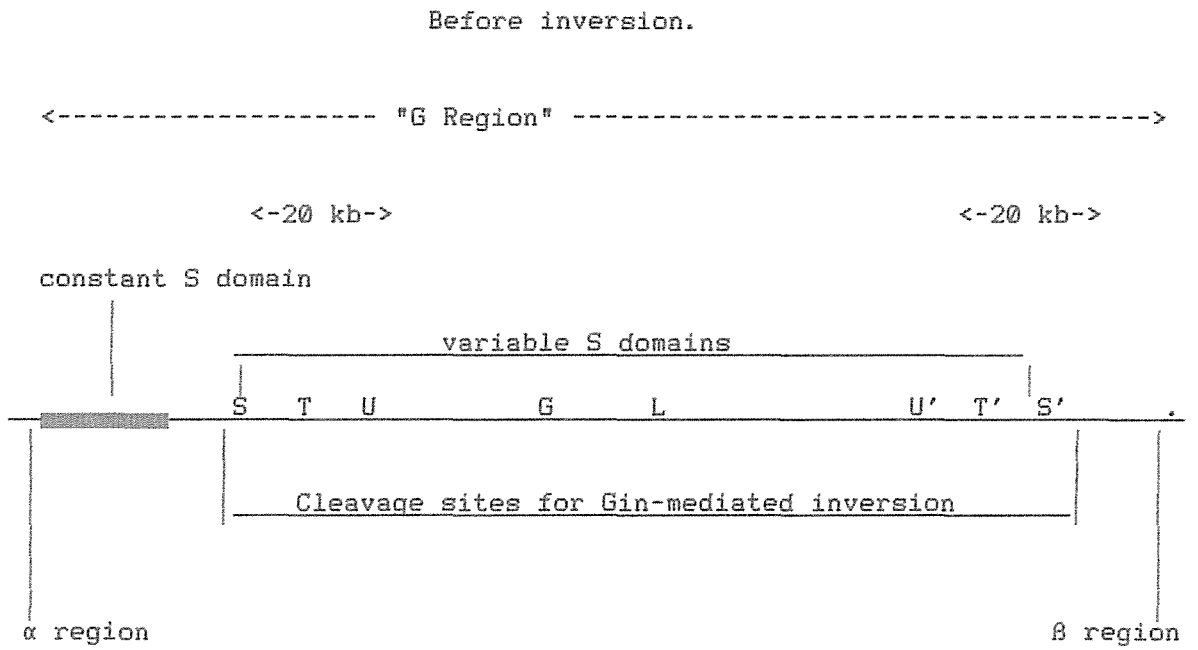


FIGURE 5 : GENE-SPLICING IN PHAGE Mu BY INVERSION OF THE G REGION.

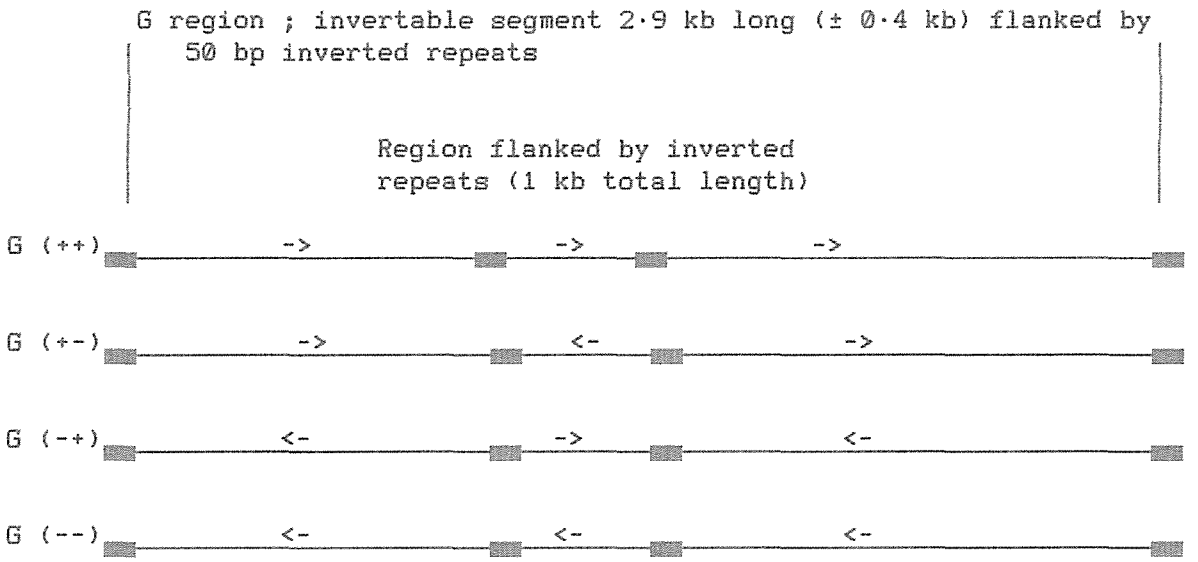
In DNA prepared from a lytic culture of phage Mu grown up on E.coli K12, less than 0·5% of the DNA shows inversion of the G-region (as detected by "G-bubbles" in heteroduplex electronmicrographs). If the phage is propagated by induction of the prophage, for example, heating a culture lysogenic for a temperature-sensitive mutant beyond the permissive temperature, then up to 50% of the phage may have inverted G-regions. It was found [Symonds and Coelho 1978] that only those phage with the G-region in a certain orientation would adsorb to the strain in which they were propagated (E.coli K12).This was taken to mean that up to half the phage produced by induction of a prophage were defective, a serious disadvantage to the phage in evolutionary terms. The phage with the G-region in the orietation which permitted adsorption to E.coli K12 were designated G+, and the orientation of the G-region in the "defective" phage was called G-. Then it was found that phage with the G-region in the G- orientation would adsorb to another host, Citrobacter freundii [Van de Putte 1980]. Phage with the G-region in one orientation would not adsorb to the host of the phage with their G-region in the other orientation. It then proved possible to isolate Gin$^-$ mutants of phage Mu, including one which was temperature sensitive (Mu cts). The Gin$^-$ strains are defective for the gene controlling inversion of the G-region and therefore display a decreased frequency of inversion. A Gin$^-$ strain with the G-region fixed in the G+ orientation will plate on E.coli K12 with an EOP of 1·0 but on Citrobacter freundii with an EOP of less than 10^{-9}. Wild type Mu propagated lytically on E.coli K12 has an EOP of 1·0 on that strain and on Citrobacter freundii it has an EOP of $2·5 \times 10^{-9}$, slightly higher than the Gin$^-$ strain. When wild type Mu is produced from E.coli K12 by induction rather than lytic propagation, approximately

50 % of the phage particles produced had their G-region in the G+ orientation and 50 % had the G-region in the G- orientation. As would be predicted from this, the EOP on Citrobacter freundii and E.coli K12 was approximately 1·0 in each case. This supported the idea that inversion of the G-region was determining the host range. It was further found that phage with the G-region in the G- orientation would adsorb to other bacterial strains (Shigella sonnei, E.coli C and others), although at frequencies greatly decreased compared to the EOP on Citrobacter freundii. This lowered EOP cannot be accounted for by restriction as some of the strains tested are naturally restrictionless. Finally, it was found that lipopolysaccharides from the cell wall of E.coli K12 would attach to phage with their G-regions in the G+ orientation, neutralising them, while lipopolysaccharides from the cell wall of E.coli C would neutralise phage with their G-regions in the G- orientation, proving that the gene products of the G-region were concerned with phage adsorption.

Using anti-sera specific to the tail proteins of phages of differing host specificity, it was found that they were all serologically distinct. A change in host range due to inversion of the G-region caused a sufficient change in the tail proteins of the phage so that it was no longer neutralised by the anti-sera specific to its parent type, but only by anti-sera specific to the phage which plated on that particular host. For example, $Mu_{k\,f}$ is not neutralised by anti-sera to $Mu_{k\,f\,k}$ and vice versa. It may be significant, however, that Mu_k is neutralised by anti-sera to W_k and vice versa; while Mu_k is not neutralised by anti-sera to W_c.

The theory was evolved from these data [Rice 1980] that the central region of the G-region, which is bounded by inverted repeats, may also be

involved in alteration of host specificity, giving rise to 4 different tail protein combinations, all with different host specificities (see figure 6). The similarities in behaviour and serology of the W-phage and phage Mu might mean that a similar system is operating in the W-phage. Against this hypothesis it must be said that inversion of the small central segment of the G-region has not been observed.



The arrows (<-/->) are merely intended to provide a reference to the direction of the DNA. They have no physical significance. Inversion of the DNA is assumed to occur by recombination within the inverted repeats. This would give rise to the figures above, depending on whether there is 1 recombination or 2.

FIGURE 6 : FOUR POSSIBLE RESULTS OF INVERSION OF GENETIC MATERIAL IN PHAGE Mu.

GENERAL MATERIALS AND METHODS.

MEDIA

All the forms of agar described in this section were dispensed aseptically into petrie dishes with 20 millilitre bases for overlay plates, or 25 millilitres for normal plates. All plates were stored at 4 °C until required.

BRAIN-HEART INFUSION MEDIA.

Broth: Difco Brain-heart infusion (dehydrated)...30 grams
Distilled water.....one litre

Agar: Difco Brain-heart infusion (dehydrated)...30 grams
Agar.....10 grams
Distilled water.....one litre

Brain-heart infusion (or BHI) is the standard medium for growth and maintainance of all the bacteria used in this thesis.

NUTRIENT AGAR FOR SLOPES

Difco Nutrient broth (dehydrated)..23 grams
Agar.....10 grams
Sodium chloride.....5 grams

This medium is ideal for long term storage of stocks. Most strains remain viable on slants of this agar for six months plus, though some Hfr strains may lose activity if not subcultured more frequently.

MINIMAL MEDIA

Broth: Dipotassium hydrogen phosphate.....7·0 grams

Potassium dihydrogen phosphate.....2·0 grams

Sodium citrate.....0·5 grams

Magnesium sulphate.....0·1 grams

Diammonium sulphate.....1·0 grams

Distilled water.....one litre

Plate agar: Make up broth double the strength actually required, since adding agar to the mixture would cause precipitation of some ions as insoluble compounds. For this reason, sterilise the double strength broth separately. After sterilisation, add from previously sterilised stocks :

Glucose (50 % w/v).....4·0 millilitres

then combine the 500 ml of double strength broth with 10 grams of agar dissolved in 500 millilitres of distilled water, sterilised separately, to produce one litre of minimal agar.

Required nutrients or antibiotics are added at the appropriate levels by rubbing onto the plate with a sterile (flamed) glass spreader.

TETRAZOLIUM SOLUTION

Triphenyl tetrazolium chloride.....0·3 grams

Sodium chloride.....0·8 grams

Potassium chloride.....0·04 grams

Distilled water.....100 millilitres

If added to overlays or normal agar plates at a concentration of 10 to 20 %, the tetrazolium is metabolised to a red compound. This is particularly useful in overlay plates to detect small plaques.

MODIFIED LENNOX MEDIA (ML)

Broth: Difco Bacto-tryptone.....15 grams

Yeast extract.....4·5 grams

Sodium chloride.....7·5 grams

Tris-chloride.....1·2 grams

Distilled water.....one litre

Adjust to pH 7·2 with 2 N HCl before sterilising. Sterilise , then add sterile stocks of :

Glucose solution (50 % w/v).....4·0 millilitres

Calcium chloride (0·05 M).....2·0 millilitres

Plate agar: Prepare Modified Lennox broth to the sterilisation stage, and add 10 grams of agar before sterilisation. Adjust to pH 7·2 as before but after sterilisation, add sterile stocks of :

Glucose solution (50 % w/v).....4·0 millilitres

Calcium chloride (0·5 M).....5·0 millilitres

The reason for the greatly increased concentration of calcium ions in the plate agar compared to the broth is the fact that the calcium precipitates when added to the hot agar. In its insoluble form the calcium is unavailable. For this reason the calcium must not be added to the broth before sterilising (autoclaving).

Overlay agar: This is prepared in exactly the same fashion as the plate agar but contains only 6·0 grams of agar per litre instead of 10. To prepare soft agar overlays for phage titrations, store in 2·5 millilitre quantities, and when needed, melt and add 1 millilitre of tetrazolium solution and 0·1 millilitre of seed bacteria.

Modified Lennox medium is a rich medium and is suitable for plate propagation of phage or for phage titration.

T-BROTH

Difco Casamino acids.....10 grams

Tris-chloride.....2·42 grams

Ammonium chloride.....1·2 grams

Sodium chloride.....1·0 grams

Potassium chloride.....0·5 grams

L-Tryptophane.....20 milligrams

Distilled water.....one litre

Add hydrochloric acid to give a final pH of 7·4

Sterilise, and after sterilisation add sterile stocks of:

Magnesium sulphate (1M).....4 millilitres

Gelatin (50 % w/v).....0·2 millilitres

Glucose (50% w/v).....10 millilitres

Thiamine-HCl [Vitamin B1] (10mM).....5·0 millilitres

Because of its high ion concentration, if all components of this medium are added together precipitation of insoluble compounds results. This medium is ideal for lytic propagation of phage.

TE BUFFER

Tris-chloride.....10 mM

Sodium-EDTA.....1 mM

Distilled water.....one litre

More correctly this is 10:1 TE buffer, the ratio referring to the ammount of Tris and EDTA respectively. This buffer is used to store DNA, and if properly purified, the DNA will not degrade appreciably for a considerable period of time.

PHOSPHATE-BUFFERED SALINE

Sodium chloride.....8·0 grams

Dipotassium hydrogen phosphate.....1·21 grams

Potassium dihydrogen phosphate.....0·34 grams

Distilled water.....one litre

This buffer is suitable for most isotonic dilutions. It is pH 7·4. Long-term storage of phage in this buffer leads to a gradual decrease in viability

MU-BUFFER

Sodium chloride.....11·7 grams

Tris-chloride.....2·4 grams

Calcium chloride.....0·1 grams

Magnesium sulphate.....4·9 grams

Gelatin.....1·0 grams

Distilled water.....one litre

This buffer is designed for storage of most phage. It is suitable for W-type phages, lambda and phage Mu.

BACTERIAL STRAINS

Strain / Substrain	Phenotype	Derivation
E. coli W:		
D_2-18	Pro⁻ Orn⁻ Leu⁻ W_c^+ W_k^+ [also Str^r mutant]	D F Bacon
D_2-18/1	Pro⁻ Leu⁻ W_c^+ W_k^+ [also Str^r mutant]	D F Bacon
D_2-18/2	Orn⁻ Leu⁻ W_c^+ [also Str^r mutant]	derived from D_2-18 by conjugation with F-2-3-27 donor.
D_2-18/3	Orn⁻ Leu⁻ W_c^+ W_k^+ [also Str^r mutant]	derived from D_2-18 by conjugation with F-2-3-27 donor.
D_2-18/4	Orn⁻ Leu⁻ W_c^+ [also Str^r mutant]	derived from D_2-18 by conjugation with F-2-3-27 donor.
D_2-18/5	Leu⁻ W_c^+ W_k^+ [also Str^r mutant]	derived from D_2-18 by conjugation with F-2-3-27 donor.
ATCC 9637	wild type W_c^+ W_k^+	D F Bacon
F-2-3-27	Pro⁻ Met⁻ His⁻ Hfr W_c^+ W_K^-	D F Bacon
F-1-4	Pro⁻ Orn⁻ Leu⁻ Met⁻ Thr⁻ W_c^+ W_k^+	D F Bacon
F-1-1-4	Pro⁻ Orn⁻ Leu⁻ Met⁻ His⁻ Thr⁻ W_c^+ W_k^+	D F Bacon
E. coli C:		
518 C	unknown requirement	W Arber
C-1-a	wild type	S Howarth
C-1-a/4	Thr⁻	A Jamieson

C-1-a/10	Leu$^-$	A Jamieson
463	Trp$^-$ Arg$^-$ Thr$^-$ StrM^r $P_1{}^r$ $T_1{}^r$	G Bertani
1463	His$^-$ $Met_5{}^-$ StrM^r Rham^{fors}	G Bertani
130	Ura$^-$ Hfr	G Bertani

E.coli K12:

PB 1395	Res$^-$	P L Berquist
AB 266	Thr$^-$ Leu$^-$ Pro$^-$ Gal$^-$ Lac$^-$ StrM^r $B_1{}^-$	D F Bacon
AB 259	Met$^-$ $B_1{}^-$ Hfr Hayes	D F Bacon
Hfr 808	Hfr 808	F Jacob
525	Thr$^-$ Leu$^-$ Lac$^-$ TonA^r Su2$^+$ Mu cts61$^+$	M Howe
526	Thr$^-$ Leu$^-$ Lac$^-$ TonA^r Su2$^+$ Mu cts 62$^+$	M Howe
AB 2569	$Arg_H{}^-$ $Met_B{}^-$ Ilv$^-$ $B_1{}^-$	EGSC
AT 253	Pro$^-$ His$^-$ $Arg_{E\,or\,H}{}^-$ $Met_A{}^-$	EGSC
AT 2535	$B_1{}^-$ Arg$^-$ His$^-$ Ade$^-$ Ura$^-$ StrM^r	EGSC

Citrobacter freundii:

D 331	unknown requirement	P van de Putte

PHAGE STRAINS

Phage	Source
T_1	D F Bacon
P_1	D F Bacon
$P_{1\,vir}$	D F Bacon
T_5	D F Bacon
Lambda	D F Bacon
Mu cts 61	M Howe
Mu cts 62	M Howe

GENERAL METHODS.

PREPARATION AND TESTING OF Hfr STRAINS.

Hfr strains of E.coli which carry the F-plasmid (or "Fertility-factor") inserted into the chromosome thus enabling the chromosomal DNA to be mobilised and transferred to compatible recipient cells with high frequencies. Under some conditions, the F-plasmid may excise and become autonomous, losing the ability to function as an efficient donor of chromosomal DNA. In this way non-Hfr revertants may accumulate in Hfr cultures during prolonged storage. It is therefore necessary to sub-culture Hfr strains on a regular basis, and test their ability to serve as donors in conjugation. This may be done by the following method:

Either streak the Hfr culture onto a new plate for single colonies, or spread a diluted sample onto a BHI plate for single colonies. About 100 colonies are desirable in either case. In either case, incubate overnight at 37°C.

Select a suitable recipient strain and subculture a single colony in BHI broth. Incubate overnight. The recipient must carry a selective marker which is transferred as an early marker by the Hfr strain.

The following day, spin down the recipient cells (4000 g for 10 minutes), discard the supernatant and resuspend in 3 millilitres of phosphate buffered saline. Spin down the cells as before, and resuspend in 1 millilitre of phosphate buffered saline, (or 0·5 millilitre if a higher concentration of recipient is required.) Spread 0·1 millilitre of this washed cell suspension onto a minimal agar plate lacking the

selective factor which is to be supplied by the Hfr. The minimal plate must also contain or lack factors to ensure counterselection of the Hfr.

Prepare 4 plates for each test; The Hfr colonies when picked should be spotted first onto the control plate, then the BHI plate and finally the test plate to avoid carrying over contaminating recipient cells.

1) Test plate (x 2). Selective minimal agar media. This is the plate on which the recipient is spread. The plate should be marked with a numbered grid so that Hfr cells which give effective conjugation can be identified. The actual conjugation is done simply by picking colonies from the overnight Hfr plates and rubbing a different one into each grid square. After overnight incubation any effective conjugation will be marked by the appearance of a group of colonies where none exists on the control. This plate should be done in duplicate.

2) Control plate. Selective minimal agar media. This is the same as the test plate but without the recipient spread on it. When the Hfr colonies are spotted onto this plate any growth must be due to mutants or contaminants. If this plate is also provided with a grid, any such colonies can be identified and discarded.

3) Brain-heart agar plate. The putative Hfr colonies should be spotted onto this plate before spotting onto the test plate! Again, this plate should be gridded to ensure that a good Hfr can be selected for continuing the stock. This check should be done to check Hfr activity before any conjugation experiments.

THE STANDARD CONJUGAL CROSS.

This is the method by which a Hfr strain is allowed to mate with a suitable recipient to transfer a desired genetic marker.

A single colony of the recipient and the Hfr (which should have been tested for its conjugative ability, as previously described) are each inoculated into 2 millilitres of BHI broth in small test-tubes.(12 or 13 millimetre tubes are ideal.) Incubate overnight at 37°C.

The next morning add a further 2 millilitres of BHI broth and incubate the cultures until the cells are in log-phase growth.

Once the cells are in log phase, they are centrifuged (4 - 5 minutes at 4000 g in a Sorvall benchtop centrifuge is sufficient) and washed twice with phosphate buffered saline. The cells are then suspended in 1 or 2 millilitres of BHI broth. 0·1 millilitres of the washed Hfr cells are mixed aseptically with 0·9 millilitres of the washed recipient cells in a sterile bijou. A test-tube is not as suitable since conjugation is an aerobic process and a bijou bottle gives a better surface-area to volume ratio for gas exchange.

Allow the cells to mate at 37°C for the desired period of time. If an interupted mating is not the aim, 1 hour is sufficient for early markers, or 2 hours for late markers.

Once mating is complete, transfer the mating mixture and the 2 parent cultures aseptically to 3 sterile test-tubes and spin down the cells as previously described. Resuspend the 3 cell suspensions in phosphate buffered saline and dilute as required. This will vary depending on the marker or markers to be selected; some late markers may need no dilution, while early markers may need 10^{-5} or greater dilution.

Then 0·1 millilitres of the dilutions are spread in duplicate on selective agar plates with a flamed glass spreader. The washed parental cells serve as controls. Incubate all plates at 37°C.

After 24 - 48 hours pick recombinant colonies and streak for single colonies, on selective plates. This reduces the error due to recombinant colonies of mixed genotype.

PLAQUE MORPHOLOGY.

Strain tested:	Plaque morphology:	
	On E.coli K12	On E.coli C
Phage W_k	Confluent, clear. Diameter about 1mm	No plaques.
Phage W_c	No plaques.	Confluent, turbid. Diameter about 2 - 3 mm.
Phage Mu	Confluent, clear. Diameter 1 - 2 mm.	No plaques
Phage P_1	Confluent, clear. Diameter 2 - 3 mm.	Confluent, clear. Diameter 2 - 3 mm.

NB. Any lysogen which is not in log-phase growth will give only a few isolated plaques.

PHAGE ASSAYS

Two methods were used to detect the presence of phage. The first is a rapid method for assaying cells for their ability to release phage. This method is particularly useful for screening large numbers of potential lysogens. However, it fails to detect lysogens releasing phage in about 2 -3 % of cases. In instances where smaller numbers of phage suspensions are to be tested, the second method offers greater reliability.

For the first method, the lysogens under test are inoculated into 2 millilitres of BHI broth and held at 37°C overnight.

The following day, 2 millilitres of fresh BHI broth are added to the tubes of lysogens and the lysogens are incubated at 37°C for 1 - 2 hours. Add 5 - 6 drops of analytical grade chloroform to each tube and incubate for at least 20 minutes. By this time all the bacteria should be killed and the tube is ready for testing

Test-plates are prepared by adding 1 millilitre of Tetrazolium solution, and 0·1 millilitre of seed bacteria to 2·5 millilitres of molten Modified Lennox overlay agar. Molten agar is held at 55°C in a water-bath. Once all the ingredients are mixed, pour the overlay onto a Modified Lennox basal plate, and allow to harden for 60 - 90 minutes. Plates can be divided into 8 sectors; 8 lysogens per plate can be tested in this way.

When the plates are ready, loopfuls of liquid are taken <u>from the top of the killed suspension</u> and spotted onto the surface of the agar overlay.

Incubate the plates overnight at 37°C. Phage plaques should be colourless against the red background of the bacterial lawn.

The second method is essentially the same, except that 0·1 millilitres of the killed suspension is added to the soft agar before pouring. This is more laborious, but it will pick up lower numbers of phage per unit volume than the first method.

ISOLATION OF LYSOGENS OF THE W-PHAGE.

A chloroform killed supernatant of E.coli W broth culture was plated on lawns of either E. coli K12 or E. coli C at a dilution sufficient to give well-spaced plaques. These were then stabbed with a sterile needle and inoculated into 2·5 millilitres of BHI broth, grown up at 37°C overnight and then streaked onto BHI agar plates. Selected single colonies which developed were grown in BHI broth and the supernatants titrated for the presence of phage W. Any suspected lysogens were then tested for immunity to phage W by spotting a drop from a BHI broth culture of the suspected lysogen onto a ML plate on which had been spread approximately 10^4 PFU of phage W. Strains lysogenic for phage W did not show any signs of lysis within the area of bacterial growth. Clones were designated lysogenic only if they produced the W-phage, and were immune to W-phage carrying the appropriate host range specificity.

ASSAYING ADSORPTION COEFFICIENTS.

The bacterial strain was grown up overnight at 37°C in BHI broth, diluted one in ten with fresh BHI broth and then grown for at least 145 minutes. The cells were then washed in phosphate-buffered saline and resuspended in BHI broth. 0·9 millilitres of this bacterial suspension, now at approximately 10^8 cells per millilitre, was added to 0·9 millilitres of a phage lysate containing 10^6-10^7 PFU per millilitre. 0·2

millilitres of 5mM Ca^{2+} was also added. Aliquots of 0·1 millilitres were removed at appropriate intervals into 9·9 millilitres of diluent containing sufficient chloroform to kill the cells. Further adsorption is prevented by the dilution. Unabsorbed phage were then titrated.

ASSAYING CELL SENSITIVITY TO PHAGE.

This is the method by which colonies were tested for sensitivity to a particular bacteriophage. Each colony to be tested is inoculated into 0·1 millilitres of BHI broth in a well of a sterilised microtitre plate. These cultures are allowed to grow for 1 - 2 hours at 37°C to give a light inoculum. If the inoculum is too heavy, the growth of the cells on the test plate may make reading the plate difficult.

Divide a Modified Lennox (ML) plate into 6 or 8 sections with horizontal lines. With a sterile loop, take a drop of culture and streak it across the plate, within the boundaries of one section. It is essential that the plate surface is sufficiently dry to prevent this line of culture from spreading into other sectors. Repeat this process until all the cultures to be tested have been streaked. Allow them to dry completely.

With a sterile loop, place a drop of the phage stock to be tested onto each streaked culture. Several phage may be tested on the one streak provided as reasonable care is taken to identify them and prevent overlapping. Alternatively, if only one phage is being used, 0·1 millilitres of the phage stock may be spread on the plate and dried before streaking the cells. Phage stocks must be at least 10^7 PFU/ml for this method to be practical.

Once spotted, the plates should be incubated overnight at 37°C. complete lysis of the cells indicates sensitivity, isolated plaques within the area that the phage was spotted may indicate restriction. No lysis at all indicates immunity, resistance, or a very strong restriction (such as that exhibited by E.coli W).

PREPARATION AND MAINTAINANCE OF PHAGE STOCKS.

This description of methods is only relevant to the preparation of small scale stocks for general laboratory use, such as testing cell sensitivity. The preparation of bulk stocks such as are required for DNA extraction is handled in a somewhat different fashion and is described in the section on DNA studies.

For small scale preparations there are two methods generally used, either tube propagation, or plate propagation. The first is the easiest and most rapid technically, but plate propagation usually gives higher titre lysates. The plate method is the one most frequently used in the work described in this thesis.

Tube propagation; for lytic phage.

0·1 millilitre of phage lysate containing at least 10^9 Plaque-Forming Units per millilitre (PFU/ml) is added to 2 millilitres of T-broth containing 5 x 10^8 sensitive log phase cells per millilitre at 37°C. The phage are allowed to adsorb to the cells for 10 minutes, at 37°C and then 8 millilitres of T-broth prewarmed to 37°C is added. The mixture is held at 37°C, and aerated aseptically. After several generations of growth the mixture will clear as the cells undergo lysis (usually in 2 - 4 hours). The mixture should then have 0·5 millilitres of chloroform added, and be held at 37°C for 20 minutes to complete lysis. After lysis is complete,

the lysate is centrifuged at 10,000 g for 10 minutes to remove bacterial debris. The lysate is titrated at this point. The lysate can be stored over chloroform at 4°C for considerable periods though phage activity may decline with prolonged storage (6 months or more).

Plate propagation; for lytic or temperate phage.

The initial preparation is the same as for tube propagation up to the pre-adsorbtion step. In advance, 5 Modified Lennox overlay tubes are melted and sensitive log-phase seed bacteria and tetrazolium added to the overlays. After pre-adsorption, 0·1 millilitres of the phage - cell mixture are added to each overlay and the overlay poured onto a ML basal plate. The plates are allowed to harden for at least 45 minutes before being incubated inverted. The plates are incubated at 37°C overnight.

Bacterial growth should be obvious in the overlay after 4 - 6 hours, and the plates are ready for harvesting when confluent lysis reaches its peak.

2 millilitres of T-broth are warmed to 37°C, then added to each plate. The agar overlay is then broken up and T-broth and agar transferred to a centrifuge tube. The resultant suspension is shaken or vortexed vigorously and 0·5 millilitres of chloroform added to kill surviving cells. The suspensions are allowed to stand for 30 minutes on the bench. The suspension is then centrifuged at 10,000 g for 10 minutes to remove bacterial debris and any agar. This procedure should yield about 10 millilitres of lysate containing about 10^{10} - 10^{11} PFU/ml. The lysate can be stored as described above.

EXPERIMENTAL SECTION 1.

EXAMINATION OF RESTRICTION IN E.COLI STRAIN W.

The phage restriction displayed by E.coli strain W is unusual in a number of respects, including the apparent lack of modification and the very high level of restriction, most likely due to the lack of modification. This restrictive ability is almost certainly due, at least in part, to a restriction system coded for by one or both of the W-prophages. This can be demonstrated in a number of ways. A type I restriction/ modification system common to the restricting E.coli strains can also be demonstrated in E.coli strain W. In certain crosses E.coli C and E.coli K12 exconjugants (where the parental recipient strains are not restrictive of the test phage) have received some E.coli W genomic material, cells which display restriction, but do not release phage may be found. These cells do not restrict phage as completely as other exconjugants which do release phage, or E.coli W itself. Cross #9 in Experimental Section 2 includes an example of this pattern of restriction. The most likely explanation for this behaviour is that the restricting non-lysogenic exconjugants have incorporated the W hsd restriction/ modification loci. This can be demonstrated by picking one of the isolated plaques which occur on the cells displaying hsd-type restriction and reinfecting these cells (putatively carrying the strain W hsd restriction/ modification loci); the reinfecting phage are no longer restricted and propagate freely. This indicates that the phage have been modified and since the parent in crosses involving E.coli C as recipient (Cross #9 referred to above) is not possessed of either modifying or

restricting abilities, these functions must have been recieved from the E.coli W donor parent. In similar crosses using an E.coli K12 recipient, test phage were grown in the E.coli K12 hosts so that the phage had been modified appropriate to the K12 strain. The K12-modified phage were restricted at the same level as phage grown in E.coli C, that is, phage which are unmodified. The putative W-strain modified phage which propagate on cells that have received the strain W hsd restriction/ modification system were equally vulnerable to restriction in control cells carrying the normal K12 hsd locus as were unmodified phage. This demonstrates that the hsd loci of strain W and strain K12 are different, although they operate in the same fashion and map at approximately the same point on the genome of their respective strains.

One of the distinguishing features of the restriction observed in E.coli W however, is the apparent lack of a modifying system. This behaviour is most likely due to a phage coded restriction system, since those exconjugants which can be shown to release W-phage display a different pattern of restriction from the hsd$^+$ cells previously described. Moreover, one which is analogous to the behavior seen in E.coli W.

A strain of lambda has been isolated which will propagate on E.coli W and on lysogens of the W-phage with an EOP of 1·0. This mutant occurs in the lambda population with a frequency of 10^{-10} on E.coli W (or 10^{-7} if the lambda population has already been modified by the W-specfic hsd system). Since progeny of this phage retain the ability to evade the W-phage coded restriction system even when propagated in non-modifying hosts, it is presumably a mutation which alters a DNA recognition site (or sites) for the phage encoded restriction endonuclease [Glover and

Aronovitch 1967, Glover et al. 1968]. If the cells lysogenic for the W-phage isolated in cross #9 (Experimental Section 2) are infected with a test phage, they display a very tight restriction and no plaques are seen. It has been shown [Pizer et al. 1968] that the failure of the phage to propagate on lysogens of the W-phage is due to restriction rather than exclusion or some other mechanism. This was determined by following the breakdown of radioactively labelled DNA in the various hosts. If lambda is propagated on E.coli C and the progeny are used to infect E.coli C lysogenic for $W_{\circ}$, it absorbs to the host cells with its typical frequency, but greater than 20 % of the lambda DNA is degraded into acid-soluble fragments in the first 10 minutes of infection. This is a similar figure to that obtained with E.coli W, and greater than the percentage of DNA breakdown when unmodified phage were used to infect E.coli K12. When phage modified by propagation on the cells carrying the W-specific hsd loci were used to infect the restricting cells lysogenic for the W-phage, no phage propagation was observed. This implies that there are at least two restriction systems present in E.coli W, one associated with the W prophage which does not appear to have an associated modification system and a strain W specific type I restriction and modification system. In the cells which have received both restriction systems, the much tighter restriction exerted by the modificationless phage-coded system will mask the effect of the hsd restriction system.

It has been shown that the modificationless restriction system found in E.coli W is associated with the presence of the W-prophage [Glover and Kerszman 1967, Pizer et al. 1968, Jamieson 1971, also see Experimental Section 2 of this thesis]. It has proved possible to put the prophage across by conjugation into normally non-restricting recipients which

thereby gain the ability to restrict. When the prophage has been removed by a second cross the restricting ability has been simultaneously lost (see Experimental Section 2). While it has been shown by previous workers, and again in this thesis (see Experimental Section 2), that both W_c and W_k can carry the genes for the restriction system, there are a number of factors which can affect the expression of prophage-coded restriction. Glover [Glover and Kerszman 1967, Glover et al. 1967] found that the W_c phage could be induced to plate on E.coli K12 instead of its normal host. This phage was designated W_c' and was found to be a mutant with an extended host range that would plate on E.coli C or E.coli K12 with equal facility. Glover also found, however, that if W_c' was used to lysogenise E.coli C, unlike W_c it did not confer restricting ability on its host. It appeared that the mutation to an extended host range also modified or inactivated the restricting ability of the prophage.

It was claimed by some workers [Glover and Kerszman 1967, Glover et al. 1967] that different strains of E.coli C, when lysogenic for the W_c prophage, showed differing levels of restriction. It was hypothesised that the prophage could occupy different sites on the host chromosome and it was postulated that the insertion site played a role in controlling the expression of the restriction system; possibly only certain sites permitted the expression of restriction. It was found, however, [Jamieson 1971] that different lysogens from a single plaque showed different behaviour. Some were restricting, and others were not. A variation in the degree of restriction was not seen. Despite the similarities between E.coli C and E.coli K12, the behaviour of the W prophage is different in each of these 2 hosts. While E.coli C lysogens of the W phage show restricting properties, E.coli K12 lysogens have never been observed to

exhibit phage-coded restriction. In view of this, and the lack of any hard evidence for Glover's hypothesis, it seems likely that the ability of the prophage to express restriction is related to the presence of an as yet unspecified host factor - present in strains of E.coli C, and in E.coli W, but not in E.coli K12. Further evidence to support this hypothesis is presented below, in Experimental Section 2 and in the Introduction and Final Discussion.

The restricting ability of the W prophage has so far only been observed directly in other strains of E.coli, such as the C or K12 strains. Because of the presence of other restriction systems in E.coli W, the study of restriction in this strain is made the more difficult. The sequential elimination of the different factors controlling the restriction systems has not proved possible so far. Glover [Glover and Kerszman 1967] was unable to isolate an E.coli W derivative cured of the W-phage by normal means, but did isolate a derivative which although it failed to release viable phage, still would not propagate the W-phage. Presumably, this was due to a defect in the prophage rather than its elimination. Other workers reported similar results [Pizer et al. 1968]. Pizer discovered that a "cured" strain (designated WS) would only propagate phage T_2 at a very low level. 99 % of the cells to which T_2 adsorbed were killed without release of phage. Only in about 1 % of cases where adsorption occurred were phage progeny released. Just as significant was the observation that T_4 would adsorb to the "cured" WS strain. Phage T_4 will not adsorb to normal E.coli W. This implies that there has been some change to the cell surface, a fact that may have importance when it is remembered that the prophage coded restriction endonuclease is most likely confined to the periplasm, in close

association to the cell surface. A second piece of evidence pointing to the involvement of cell surface structures is the fact that the ability of T_4 to adsorb to strain WS is decreased when the host WS cells are made lysogenic for the W-phage. This indicates that the presence of the W-prophage affects the cell surface structures in some way, though there is no evidence as yet of any mechanism by which this might occur. WS lysogens of the W-phage (WS(w)) could be shown [Pizer et al. 1968] to degrade invading T_2 DNA in 66 % of cases studied, and none of the WS(w) cells propagated phage T_2.

Finally, a further mutant of E.coli W was observed by Pizer's group after treatment with nitroguanosine. This strain produced viable phage particles which could infect E.coli C and, as previously seen, could cause lysogens of E.coli C to express the typical phage-coded restriction system. However, in the E.coli W mutant, the phage coded restriction system was not expressed. Since the progeny phage from these restrictionless E.coli W cells did not appear to be defective in any way, the most likely reason for the loss of restriction in E.coli W was the inactivation of the gene(s) responsible for the postulated host factor necessary for effective restriction.

EXPERIMENTAL SECTION 2

ANALYSIS OF W-PHAGE CODED RESTRICTION.

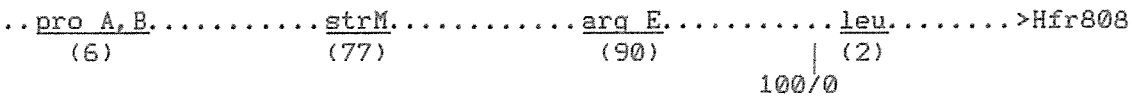
It was proposed that, as the existence in E.coli strain W of more than 1 type of restriction had been demonstrated, a valid means of examining these systems might be to remove genetic loci coding for restriction (and modification in the case of the hsdw loci) independantly and sequentially by conjugation and segregation; in this way each system might be examined in isolation in the host (E.coli W), in which it normally operates. This information would also shed light on the linkage relationships of each of the genetic loci coding for restriction. To this end, the following conjugation crosses were performed in the manner outlined in the general methods section. To aid in understanding each cross has been illustrated with a basic map (not to scale) indicating the relative positions of the markers carried by each strain, where these are known. The underlined letters are the standard genetic nomemclature for the loci. The numbers in parentheses represent map positions in minutes on the E.coli K12 genetic map. [Bachmann 1983]

Conjugation cross #1

A K12 Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by the incorporation of Streptomycin at 200 micrograms per ml. Exconjugants (Leu^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agent were supplied at 50 micrograms/ millilitre.

Hfr 808 (K12), P.O. 5 minutes : Wild type

F^- D_2-18 (W), : Leu^- Orn^- Pro^- W_c^+ W_k^+ $StrM^r$



Marker selected for : $Leucine^+$

Number of recombinants screened : 60

Secondary character (unselected) : Observed number and frequency :

Orn^+ (arg E)	2	3·3 %
Pro^+	0	0 %
Phage W_c non-releasing (W_c^-)	0	0 %
Phage W_k non-releasing (W_k^-)	12	20 %
Phage P_1 non-restricting (res^-)	0	0 %

The purpose of this conjugation was to eliminate the W_c prophage by recombination since there was prior evidence [Jamieson 1971] of significant linkage of the W_c prophage to the Leucine gene. This was not successful. A relatively low number of selected recombinants was obtained and this was probably due, in part, to restriction of the donor DNA. The most interesting result from this cross was the observation that the W_k prophage was eliminated in 20 % of recombinants, an unexpected result if the insertion site of the W_k prophage in fact lies between the F insertion site of Hfr808 the pro A,B loci, as suggested by earlier

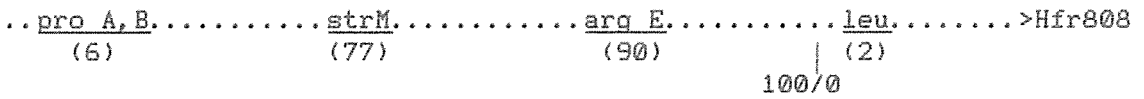
workers [Jamieson 1971]. The frequency of elimination of the W_k prophage implies that it maps at a site adjacent to genes transferred as early markers by strain Hfr 808.

Conjugation cross #2

A K12 Hfr strain was crossed with a W strain recipient (F-). The Hfr was counterselected by the incorporation of Streptomycin at 200 micrograms per ml. Exconjugants (Orn^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agent were supplied at 50 micrograms/ millilitre.

Hfr 808 (K12), P.O. 5 minutes : Wild type

F^- D2-18 (W) : Leu^- Orn^- Pro^- Wc^+ Wk^+ $StrM^r$



Marker selected for : $Ornithine^+$ (arginine E)

Number of recombinants screened : 60

Secondary character (unselected) :	Observed number	and frequency :
Leu^+	2	3·3 %
Pro^+	0	0 %
Phage W_c non-releasing (W_c^-)	0	0 %
Phage W_k non-releasing (W_k^-)	1	0·16 %
Phage P_1 non-restricting (res^-)	0	0%

This cross was again designed to eliminate the W_c prophage since as mentioned (Conjugation cross #1) there was reason to suppose that the W_c prophage mapped in the region of the arginine I marker. The Ornithine requirement is due to a mutation in the more distal arginine E locus and by

selecting for this more distant marker the chances of detecting crossing over in the region between arg E and arg I might be improved. The observation that the W_c prophage was not eliminated will be discussed later. As noted under cross #1, restriction is a likely reason for the recovery of a lower number of Orn$^+$ recombinants than was expected. The W_k^- phenotype was observed in this cross as well as in cross #1, adding evidence to the hypothesis that the W_k prophage insertion site lies close to genes transferred in the early stage of conjugation.

Conjugation cross #3

A K12 Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by the incorporation of Streptomycin at 200 micrograms per ml. Exconjugants (Pro^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agent were supplied at 50 micrograms/ millilitre.

Hfr AB259 (K12), P.O. 97 minutes : Met^- B_1^-

F^- D_e-18 (W) : Leu^- Orn^- Pro^- W_c^+ W_k^+ $StrM^r$

<Hfr Hayes....leu.....strM.....met F arg E thiA,B,C.....pro A,B..
(97) (2) (77) (89) (90) 100/0 (6)

Marker selected for : Proline$^+$

Number of recombinants screened : 60

Secondary character (unselected) :	Observed number and frequency :	
Leu^+	18	30 %
Phage W_c non-releasing (W_c^-)	0	0 %
Phage W_k non-releasing (W_k^-)	37	61·6 %
Phage P_1 non-restricting (res^-)	0	0 %

After the failure to observe the removal of the W_e prophage in Conjugation cross #2, this conjugation was performed using a Hfr strain which transfers the genetic material in the opposite direction to the Hfr 808 strain employed in crosses #1 and #2 and which would include the sites to which the W_k prophage and possibly the W_e prophage had been tentatively mapped [Jamieson 1971]. As predicted the W_k prophage was eliminated with a high frequency, but the W_e prophage was not removed in any instance. Since the W_k prophage has been removed in a number of exconjugants, the disappearance of the W_e cannot be masked by phage produced by the W_k prophage by genetic switching in these cases. It is possible the W_e prophage may not be present in the area covered by the Hfr or its disappearance may be masked by the presence of another W_e prophage located elsewhere on the host genome.

Conjugation cross #4

A W Hfr strain was crossed with a C strain recipient (F^-). The Hfr was counterselected by the omission of Histidine and Methionine from the agar. Exconjugants (Thr^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agents were supplied at 50 micrograms/ millilitre.

Hfr F-2-3-27 (W), P.O. 5 minutes : His^- Met^-

F^- C-1-a/4 (C) : Thr^-

.....his A-I.....met F.....thr.....>Hfr808
(44) (89) 100/0

Marker selected for : $Threonine^+$

Number of recombinants screened : 60

Secondary character (unselected) :	Observed number and frequency :	
Phage W_e Releasing (W_e^+)	36	60 %
Phage P_1 Restricting (res^+)	40	66·7 %

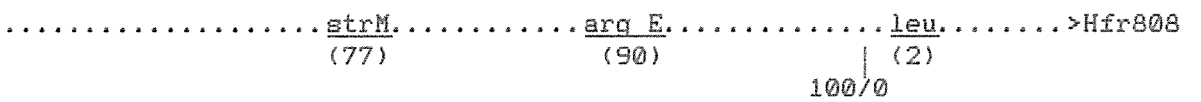
The approximate 7 % of the sample which were restricting but did not carry the W_e prophage were shown to be expressing the E.coli W hsd type restriction. Presumably some of the W_e^+ cells are also carrying the hsdW loci but these were masked by the presence of the more active W-prophage coded restriction system.

Conjugation cross #5

A K12 Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by the incorporation of streptomycin at 200 micrograms per ml. Exconjugants (Orn^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agents were supplied at 50 micrograms/ millilitre.

Hfr 808 (K12), P.O. 5 minutes : wild type.

F^- D2-18/2 (W) : Leu^- Orn^- Wc^+ $StrM^r$



Marker selected for : $Ornithine^+$

Number of recombinants screened : 20

Secondary character (unselected) :	Observed number and frequency :	
Leu^+	1	5 %
Phage W_c non-releasing (W_c^-)	0	0 %
Phage W_k non-releasing (W_k^-)	20	100 %
Phage P_1 non-restricting (res^-)	0	0 %

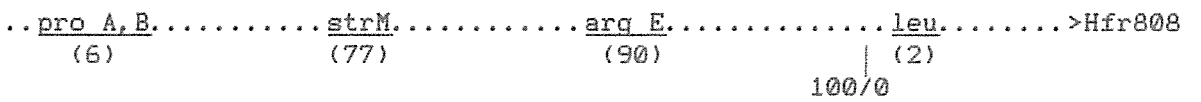
This cross was done to give further support to the assumption that the W_k prophage does not undergo genetic switching to mask the disappearance of the W_c prophage by releasing phage with the W_c phenotype. The apparent persistence of the W_c prophage leaves open the possibility of another prophage located at a second site on the host genome.

Conjugation cross #6

A K12 Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by the incorporation of streptomycin at 200 micrograms per ml. Exconjugants (Orn^+ Leu^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agents were supplied at 50 micrograms/ millilitre.

Hfr 808 (K12), P.O. 5 minutes : wild type.

F^- D_2-18 (W) : Leu^- Orn^- Pro^- W_c^+ W_k^+ $StrM^r$



Markers selected for : $Ornithine^+$ and $Leucine^+$

Number of recombinants screened : 60

Secondary character (unselected) :	Observed number	and frequency :
Phage W_c non-releasing (W_c^-)	0	0 %
Phage W_k non-releasing (W_k^-)	7	11·6 %
Phage P_1 non-restricting (res^-)	0	0 %
Pro^+	0	0 %

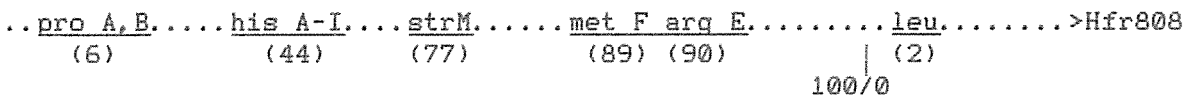
This cross was a further attempt to eliminate the W_c prophage by requiring multiple crossing over in the area of interest and thus increase the chances of eliminating the W_c prophage.

Conjugation cross #7

A W Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by the omission of Histidine and Methionine from the agar. Exconjugants (Pro^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agents were supplied at 50 micrograms/ millilitre.

Hfr F-2-3-27 (W), P.O. 5 minutes : His^- Met^-

F^- D_2-18 (W) : Leu^- Orn^- Pro^- W_o^+ W_k^+ $StrM^r$



Marker selected for : $Proline^+$

Number of recombinants screened : 12

Secondary character (unselected) :	Observed number and frequency :	
Phage W_k non-releasing (W_k^-)	4	33·3 %
Hfr_{sss}^+	5	41·6 %

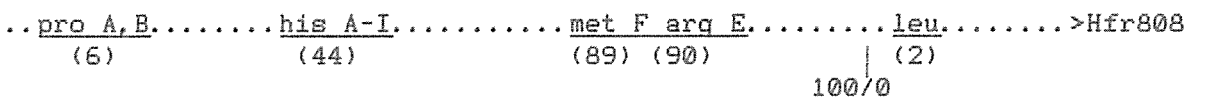
It was previously thought that the W_k prophage insertion site mapped between the insertion site of the F plasmid in Hfr808 and the pro A gene, It was therefore a surprise to find that a relatively large proportion of the exconjugants were still W_k^+, revealing a looser linkage to the pro A locus than previously observed [Jamieson 1971]. Still more important was the observation that as predicted on the basis of crosses #1 and #2, the prophage mapped counter-clockwise of the Hfr insertion site, and was transferred as an early marker by a W_k^+ Hfr derived from this cross. This proves that the W_k prophage insertion site must lie clockwise of the Hfr insertion site.

Conjugation cross #8

A W Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by the omission of Histidine and Methionine from the agar. Exconjugants (Pro^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agents were supplied at 50 micrograms/ millilitre.

Hfr F-2-3-27 (W), P.O. 5 minutes : His^- Met^-

F^- D_2-18 (W) : Leu^- Orn^- Pro^- W_c^+ W_k^+



Marker selected for : $Proline^+$

Number of recombinants screened : 20

Secondary character (unselected) :	Observed number	and frequency :
Phage W_c non-releasing (W_c^-)	0	0 %
Phage W_k non-releasing (W_k^-)	5	25 %
Phage P_1 Restricting (res^+)	20	100 %
Hfr_{sss}^+	7	35 %

This cross was made to extend the results of Conjugation cross #7, and to derive a streptomycin sensitive W_k^+ Hfr. Such a strain would make it easier to test whether the W_k prophage does in fact map to a site counter-clockwise to the Hfr808 insertion site. In that event such an Hfr would transfer the W_k prophage as an early marker. See cross #9.

Conjugation cross #9

A W Hfr strain was crossed with a C strain recipient (F^-). The Hfr was counterselected by the omission of Ornithine and Leucine from the agar. Exconjugants (Thr^+) were screened for expression of unselected donor characteristics. All nutritional requirements other than the selective agents were supplied at 50 micrograms/ millilitre.

Hfr D_2-18/3 (W), P.O. 5 minutes, : Leu^- Orn^- W_c^+ W_k^+ (Derived from Conjugation cross #8)

F^- C-1-a/4 : Thr^- Nal^r

.....nal B.....arg E....thr...leu.....>Hfr808
(58) (90) (100/0)(2)

Marker selected for : $Threonine^+$

Number of recombinants screened : 60

Secondary character (unselected) :	Observed number	and frequency :
Phage W_c Releasing (W_c^+)	12	20 %
Phage W_k Releasing (W_k^+)	34	73·3 %
Phage P_1 Restricting (res^+ ; W-phage type)	42	70 %
Phage P_1 Restricting (res^+ ; hsdW type)	8	13·3 %
Leu^+	48	80 %

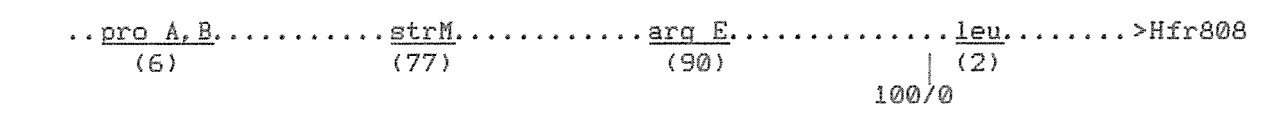
This mating was carried out using a W_k^+ Hfr derived in cross #8 and interupted after 15 minutes to reduce transferral of the W_c prophage. The cells were washed extensively to minimise infection of the recipient by phage released into the media from the Hfr strain. This cross demonstrates that the W_k prophage is transferred as an early marker, since it does not infect the E.coli C recipient and therefore must have been transferred by conjugation. For comment on the transferral of the hsdW restriction system, see the notes under Conjugation cross #4.

Conjugation cross #10

A K12 Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by incorporation of Streptomycin at 200 micrograms per ml of agar. Exconjugants (Pro^+) were screened for expression of unselected donor characteristics. All nutritional requirements were supplied at 50 micrograms/ millilitre.

Hfr 808 (K12) P.O. 5 minutes : wild type.

F^- D_2-18 : Leu^- Orn^- Pro^- W_c^+ W_k^+ $StrM^r$



Marker selected for : $Proline^+$

Number of recombinants screened : 20

Secondary character (unselected) :	Observed number and frequency :	
Phage W_c non-releasing (W_c^-)	0	0 %
Phage W_k non-releasing (W_k^-)	2	10 %
Hfr_{808}^+	3	15 %

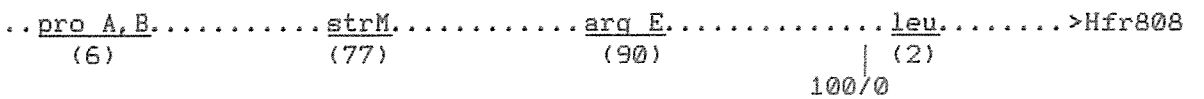
This cross was done to reduce the possibility that a significant difference in gene order exists between the F-2-3-27 Hfr and the E.coli K12 Hfr strain 808. A D_2-18 Hfr derived in this cross gave similar results when Conjugation cross #9 was repeated.

Conjugation cross #11

A W Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by the omission of Ornithine from the agar. Exconjugants (Pro^+) were screened for expression of unselected donor characteristics. All nutritional requirements except selective agents were supplied at 50 micrograms/ millilitre.

Hfr D_2-18/4 P.O. 5 minutes : Leu^- Orn^- W_e^+ $StrM^r$

F^- D_2-18/1 : Leu^- Pro^- W_e^+ W_k^+



Marker selected for : $Proline^+$

Number of recombinants screened : 12

Secondary character (unselected) :	Observed number and frequency :	
Phage W_k non-releasing (W_k^-)	2	16·7 %
Hfr_{sss}^+	3	25 %

This cross was an attempt to provide an Hfr strain in which genetic material from other strains had been minimised. The Hfr strain derived in this cross behaved in a similar fashion to analogous Hfr strains derived previously.

Conjugation cross #12

A W Hfr strain was crossed with a W strain recipient (F^-). The Hfr was counterselected by the omission of Histidine and Methionine from the agar. Exconjugants (Pro^+) were screened for the expression of unselected donor characters. All nutritional requirements except the selective agents were supplied at 50 micrograms/ millilitre.

Hfr F-2-3-27 P.O. 5 minutes : Met^- His^- W_c^+ W_k^-

F^- D_s-18/1 : Leu^- Pro^- W_c^+ W_k^+

..pro A,B.....his A-I.....met F.....leu.....>Hfr808
(6) (44) (89) (2)
100/0

Marker selected for : Proline$^+$

Number of recombinants screened : 15

Secondary character (unselected) :	Observed number and frequency :	
Phage W_k non-releasing (W_k^-)	3	20 %
Hfr_{sss}^+	3	20 %

This cross was performed as a check upon the results of Conjugation cross #11, and with the same intention.

These 12 crosses, which were focussed mainly on the W_k prophage, indicated that the W_k insertion site lies at approximately 5 minutes on the E.coli K12 genetic map. More detailed mapping is discussed in Experimental section 3. The observations made regarding restriction are similar to those of previous workers [Jamieson 1971]. This aspect will be discussed in the following section.

A number of additional crosses were carried out with the W_c prophage as the main point of interest, and these immediately follow.

Conjugation cross #13

A W Hfr strain was crossed with a K12 strain recipient (F^-). The Hfr was counterselected by the omission of Histidine and Methionine from the agar. Exconjugants (Leu^+) were screened for expression of unselected donor characteristics. All nutritional requirements except selective agents were supplied at 50 micrograms/ millilitre.

Hfr F-2-3-27 P.O. 5 minutes : Met^- His^- W_c^+ W_K^-

F^- AB266 : B_1^- Gal^- Lac^- Leu^- Pro^- Thr^- $StrM^r$

..pro A,B..lac....his A-I...strM....met F thi A-C...thr....leu....>Hfr808
(6) (8) (44) (77) (89) (90) (100/2) (2)

Marker selected for : $Leucine^+$

Number of recombinants screened : 113

Secondary character (unselected) :	Observed number and frequency :	
Phage W_c Releasing (W_c^+)	83	73·5 %
Thr^+	102	90·3 %
Phage P_1 restricting (res^+ ; hsdW type)	101	88·3 %
Phage P_1 restricting (res^+ ; W-phage type)	0	0 %

The most interesting feature of these results is the fact that, as reported by other workers, [Glover and Kerszman 1967, Jamieson 1971] the E.coli K12 host does not restrict P_1 (modified against the K12 restriction endonuclease) when carrying the W_c-prophage. However the E.coli K12 strain does restrict K12-modified P_1 when it recieves the hsdW restriction/ modification system. This implies that some host factor is required by the W-prophage mediated restriction system to be effective, a factor which is present in E.coli C and in E.coli W, but is not present in E.coli K12. E.coli K12 was chosen as a recipient in these crosses since the W_c phage will not adsorb to it ; any appearance of the W_c

prophage in the exconjugants can be confidently attributed to recombination.

Conjugation cross #14

A W Hfr strain was crossed with a K12 strain recipient (F^-). The Hfr was counterselected by the omission of Histidine and Methionine from the agar. Exconjugants (Ura^+) were screened for expression of unselected donor characteristics. All nutritional requirements except selective agents were supplied at 50 micrograms/ millilitre.

Hfr F-2-3-27 P.O. 5 minutes : Met^- His^- W_c^+ W_K^-

F^- AT2535 : $B1^-$ Arg^- His^- Ade^- Ura^- $StrM^r$

..pro A,B.....his A-I..strM...met F thi A-C..pyr B...leu.....>Hfr808
(6) (44) (77) (89) (90) (97) | (2)
100/0

Marker selected for : $Uracil^+$ (pyrimidine B)

Number of recombinants screened : 60

Secondary character (unselected) :	Observed number	and frequency :
Phage W_c Releasing (W_c^+)	42	70 %
Vitamin B_1^+	29	48·3 %
Phage P_1 restricting (res^+ ; hsdW type)	40	66·7 %
Phage P_1 restricting (res^+ ; W-phage type)	0	0 %

Linkage of the W_c prophage to the pyrimidine B marker is indicated in this cross. Since the B_1 gene locus is linked to the pyrimidine B marker it should also exhibit linkage to the W_c prophage. Thus, if a cross was performed to eliminate the B_1 marker, it should remove the W_c prophage with a reasonable frequency (see Conjugation cross #15).

Conjugation cross #15

A K12 Hfr strain was crossed with a K12 strain recipient (F^-). The Hfr was counterselected by the incorporation of Streptomycin at 200 micrograms per ml of agar. Exconjugants (B_1^+) were screened for expression of unselected donor characteristics. All nutritional requirements except selective agents were supplied at 50 micrograms/ millilitre.

Hfr 808 P.O. 5 minutes : Wild type (K12)

F^- AT2535/1 : B_1^- Arg^- His^- Ade^- $StrM^r_A$ W_e^+ (derived from cross #14)

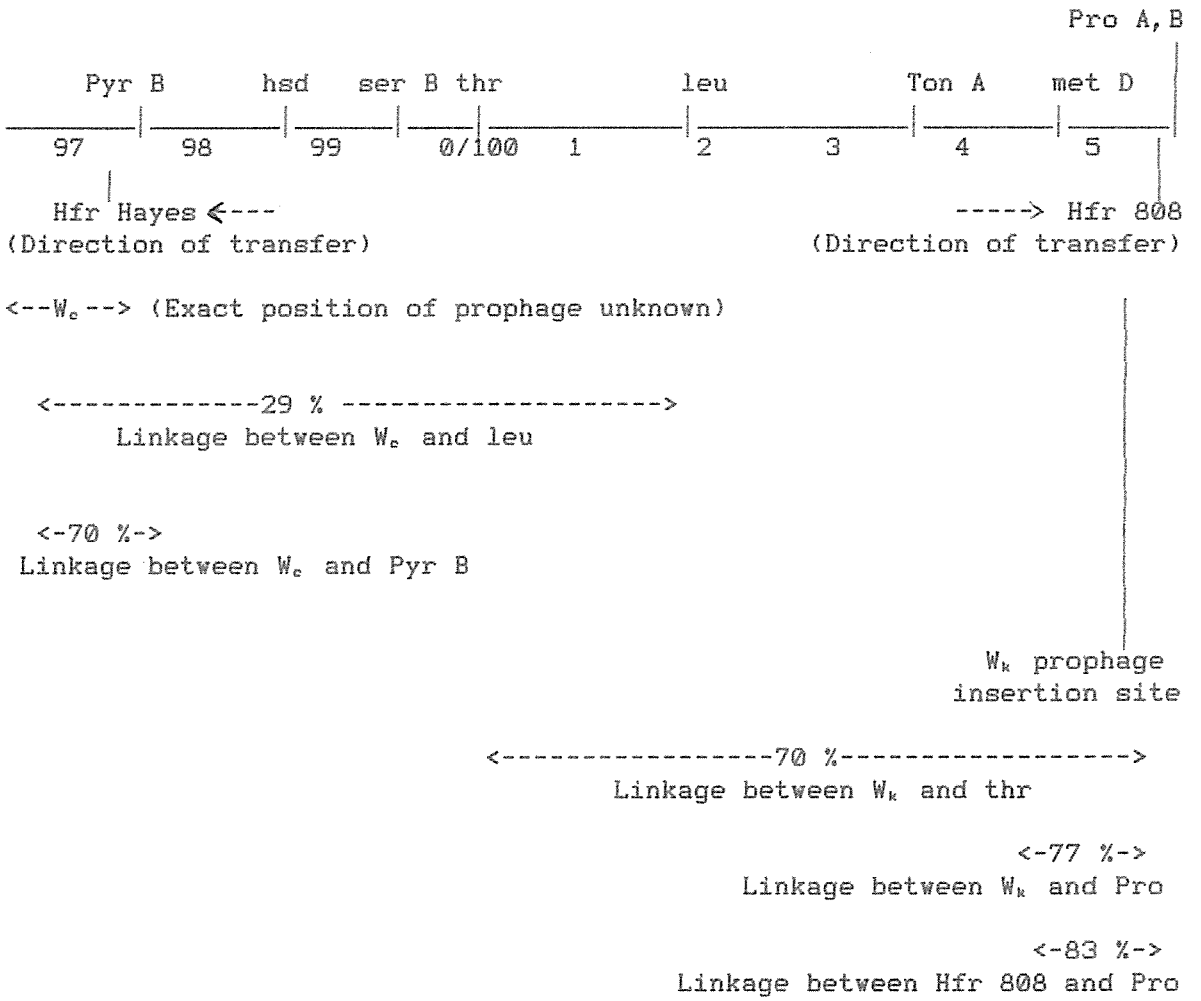
..pro A,B.....his A-I....strM.....met F thi A-C..hsdW...leu.....>Hfr808
(6) (44) (77) (89) (90) (99) | (2)
100/0

Marker selected for :B_1^+

Number of recombinants screened : 60

Secondary character (unselected) :	Observed number and frequency :	
Phage W_e non-releasing (W_e^-)	6	10 %
Phage P_1 non-restricting (res^- ; hsdW type)	11	18·3 %
Phage P_1 non-restricting (res^- ; W-phage type)	60	100 %

This cross was performed with the goal of crossing out the W_e prophage. It was observed that the majority of the exconjugants continued to release W_e type phage. This contrasts with previous crosses (crosses #1, #2, #5 and #6) where the W_e prophage has never been observed to be removed. If it were accepted that there were two prophages coding for the W_e phage in E.coli W, then these results would be explained; two events would be necessary to derive a strain no longer carrying (releasing) the W_e phage.



N.B. Results are not drawn to scale.

FIGURE 7 : SUMMARY OF LINKAGE RESULTS FROM EXPERIMENT 2.

2) W-Phage Coded Restriction in Other Strains of E.coli.

With the failure of the conjugation experiments detailed above to yield an exconjugant of E.coli which no longer released the W_e phage, it was not possible to examine the restriction patterns attributable to each prophage in that strain, independantly of one another.

It was therefore decided that the restrictive ability of the two prophages and of the E.coli W hsd restriction/ modification system might be examined by transferral to another host where they could be expressed either in combination or in isolation. Another series of crosses were performed, this time with the purpose of transferring a single type of prophage, or both prophages, and/ or the E.coli W hsd restriction system so that the operation of these systems could be observed together and singly. Exconjugants were picked and tested for release of phage and then for their ability to restrict phages P_1 and lambda at different phage titres.

The lambda and P_1 phage used to test for restrictive ability were grown on a modifying E.coli K12 strain and are thus not affected by the E.coli K12 hsd restriction system which may be present in some strains. The restricting phenotypes expressed towards phages P_1 and Lambda have been recorded as follows:

+	positive restricting ability ; no test phage propagation.
+/-	decreased restricting ability ; isolated plaques of the test phage
-	no or low restricting ability ; confluent lysis by the test phage.

Various stock strains were tested to determine their pattern of restriction towards phage P_1 and Lambda; phage released by these strains were also scored using E.coli C and E.coli K12 as hosts.

Stock Culture	Phage present in culture supernatant	Restriction pattern: Lambda PFU/ml		P_1 PFU/ml	
		3×10^8	3×10^6	10^8	10^6
F-2-3-27	Isolated plaques on E.coli C No plaques on E.coli K12	+	+	+	+
D_2-18	Isolated plaques on E.coli C Isolated plaques on E.coli K12	+	+	+	+
D_2-18/1	Isolated plaques on E.coli C Isolated plaques on E.coli K12	+	+	+	+
D_2-18/4	Isolated plaques on E.coli C No plaques on E.coli K12	+	+	+	+
D_2-18/3	Isolated plaques on E.coli C Isolated plaques on E.coli K12	+	+	+	+
C-1-a/4	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-
PB 1395	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-
AB 266	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-
518C	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-
D 331	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-

The concentrations of phage used in this experiment were sufficient to give confluent lysis of totally sensitive bacteria.

In the following cross a W_c^+/W_k^+ Hfr (P.O. 5 minutes) was used to run the W_k prophage into an E.coli C recipient.

Cross #I : D_2-18/3 Hfr x C-1-a/4 (Thr^-)

.....arg E.....thr.....leu.....>Hfr808
(90) (100/0) (2)

Marker selected for : $Threonine^+$

Total number of exconjugants tested : 36

Number of Exconjugants	Phage present in culture supernatant	Restriction pattern: Lambda PFU/ml		P_1 PFU/ml	
		3×10^8	3×10^6	10^8	10^6
5	Confluent plaques on E.coli C Isolated plaques on E.coli K12	+	+	+	+
16	Isolated lysis on E.coli C Isolated plaques on E.coli K12	+	+	+	+
1	No plaques on E.coli C Isolated plaques on E.coli K12	+	+	+	+
3	Isolated plaques on E.coli C No plaques on E.coli K12	+	+	+	+
5	No plaques on E.coli C No plaques on E.coli K12	-	+/-	-	+/-
6	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-

This cross demonstrates that the presence of a single W prophage is sufficient to confer W-type restriction on the E.coli recipient. Five exconjugants were shown to be carrying the hsdW loci in the absence of either the W_e or W_k prophage. This cross (as well as crosses #1 and #2) demonstrates that the presence of either prophage is associated with the W-type restriction, implying that both W-page carry the genes coding for the restriction endonuclease. This supposition is supported by the DNA restriction digests obtained in Experimental section 4

In the following cross an Hfr is used which has been shown to be free of the W_k prophage.

Cross # II : F-2-3-27 Hfr x C-1-a/4 (Thr^-)

.....his A-I.....met .....thr.....>Hfr808
(44) (89) 100/0

Marker selected for : $Threonine^+$

Total number of exconjugants tested : 15

Number of Exconjugants	Phage present in culture supernatant	Restriction pattern: Lambda PFU/ml		P_1 PFU/ml	
		3×10^8	3×10^6	10^8	10^6
6	Confluent lysis on E.coli C No plaques on E.coli K12	+	+	+	+
2	Isolated plaques on E.coli C No plaques on E.coli K12	+	+	+	+
2	No plaques on E.coli C No plaques on E.coli K12	-	+/-	-	+/-
5	No plaques on E.coli C	-	-	-	-

No plaques on E.coli K12

The expected phenotypes are present and this supports the conclusion that the presence of the W_e prophage by itself is sufficient to endow the cell with the typical W-type restriction.

The following cross serves as a check on the results obtained in cross #II; different but analogous strains are used.

Cross #III : D_e-18/3 Hfr (W_k^+ derivative) x C-1-a/10 (Leu^-)

.....arg E.....thr.....leu.....>Hfr808
(90) (100/0) (2)

Marker selected for : $Leucine^+$

Total number of exconjugants tested : 24

Number of Exconjugants	Phage present in culture supernatant	Restriction pattern: Lambda PFU/ml		P_1 PFU/ml	
		3×10^8	3×10^6	10^8	10^6
8	Confluent lysis on E.coli C No plaques on E.coli K12	+	+	+	+
3	Isolated plaques on E.coli C Isolated plaques on E.coli K12	+	+	+	+
2	Isolated plaques on E.coli C No plaques on E.coli K12	+	+	+	+
2	No plaques on E.coli C No plaques on E.coli K12	-	+/-	-	+/-
9	No plaques on E.coli C	-	-	-	-

No plaques on E.coli K12

The same conclusion can be drawn from this cross as the former. This is that the presence of the W_e prophage alone is sufficient to cause the cell to display a tight restriction of phages lambda and P_1. The presence of the hsdW restriction/ modification loci in the econjugants was observed again.

Cross #IV : F-2-3-27 Hfr x AB266 (gal$^-$ lac$^-$ Leu$^-$ Pro$^-$ Thr$^-$ StrM^r)

..pro A,B..lac....his A-I...strM....met F thi A-C...thr....leu....>Hfr808
(6) (8) (44) (77) (89) (90) (100/2) (2)

Marker selected for : Leucine$^+$

Number of exconjugants : 18

Number of Exconjugants	Phage present in culture supernatant	Restriction pattern: Lambda PFU/ml		P_1 PFU/ml	
		3×10^8	3×10^6	10^8	10^6
9	Isolated plaques on E.coli C No plaques on E.coli K12	-	-	-	-
2	Isolated plaques on E.coli C No plaques on E.coli K12	+/-	+/-	+/-	+/-
6	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-
1	No plaques on E.coli C No plaques on E.coli K12	+/-	+/-	+/-	+/-

Note that in this experiment the phage used to test the restricting abilities of the exconjugants were propagated in a host with the E.coli K12 specific modifying system, and were therefore unaffected by exconjugants carrying the E.coli K12 restriction system. The phages remain sensitive to the E.coli W hsd restriction, however, and it is presumably the transferral of this marker that is responsible for the low level of phage propagation on some of the exconjugants. The interesting conclusion to be drawn from this cross is that the possession of the W_o prophage does not confer restricting ability on this strain E.coli strain K12.

The following cross was done to ensure the conclusion drawn in cross #IV was not based on the results of a cross with a single strain and to derive a strain for further use.

Cross #V : F-2-3-27 Hfr x AT 2535 (B_1^- arg^- his^- ade^- ura^- $StrM^r$)

..pro A,B.....his A-I..strM...met F thi A-C..pyr B...leu.....>Hfr808
(6) (44) (77) (89) (90) (97) | (2)
100/0

Marker selected for : uracil$^+$ (pyrimidine B)

Number of exconjugants : 18

Number of Exconjugants	Phage present in culture supernatant	Restriction pattern: Lambda PFU/ml		P_1 PFU/ml	
		3×10^8	3×10^6	10^8	10^6
8	Isolated plaques on E.coli C No plaques on E.coli K12	-	-	-	-
2	Isolated plaques on E.coli C No plaques on E.coli K12	+/-	+/-	+/-	+/-
5	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-
3	No plaques on E.coli C No plaques on E.coli K12	+/-	+/-	+/	+/-

Confirming the results of cross #IV, this cross again demonstrated that the presence of the W_e prophage in an E.coli K12 is not sufficient to confer W-type restricting ability on the host. Some other condition must be met, presumably some host factor not present in E.coli K12. In this cross as in others, the presence of the hsdW restriction/ modification system is expressed.

The aim of this cross was to attempt to remove the W_c prophage by recombination and to examine the linkage of W_c and hsdW to the selected marker.

Cross #VI : Hfr 808 x AT 2535/1 derivative from cross #V (B_1^- arg$^-$ his$^-$ ade$^-$ W_c^+ StrM^r)

..pro A,B.....his A-I..strM...met F thi A-C....hsdW....leu.....>Hfr808
(6) (44) (77) (89) (90) (99) | (2)
100/0

Marker selected for : B_1^+

Number of exconjugants : 20

Number of Exconjugants	Phage present in culture supernatant	Restriction pattern: Lambda PFU/ml		P_1 PFU/ml	
		3 x 10^8	3 x 10^6	10^8	10^6
14	Isolated plaques on E.coli C No plaques on E.coli K12	-	-	-	-
2	Isolated plaques on E.coli C No plaques on E.coli K12	+/-	+/-	+/-	+/-
1	No plaques on E.coli C No plaques on E.coli K12	-	-	-	-
3	No plaques on E.coli C No plaques on E.coli K12	+/-	+/-	+/-	+/-

The B_1 marker is somewhat more distant than would be ideal, but despite this, 4 exconjugants were isolated which appeared to have lost the prophage. In addition, a decreased number of these exconjugants gave a response which we would expect if they were also carrying the hsdW

restriction/ modification system. These results provide some evidence for a W_c prophage mapping in the region around 95 minutes on the E.coli genome. Since these exconjugants do not display the W-phage type restriction even when the W-prophages are present, unfortunately it cannot provide evidence of the removal of prophage and restriction simultaneously.

EXPERIMENTAL SECTION 3

MAPPING THE SITE OF INSERTION OF THE Wk PROPHAGE IN E.COLI STRAIN W.

The initial approach was to map the W_k prophage by interupted matings, that is, to determine the time at which selected markers, chosen for their position relative to one another on the E.coli genome, were transferred by a selected Hfr strain. Earlier experiments (see Experimental sections 1 and 2) had indicated that there is considerable homology between the gene maps of E.coli K12 and E.coli W. The work described in Experimental section 2 also indicated that there might be reason to doubt the position tentatively assigned to the W_k prophage on the gene map of E.coli W, since the W_k prophage could be transferred as an early marker by a strain W Hfr derived from the E.coli K12 strain, Hfr 808.

The method used was to prepare cultures of the Hfr and F^- strains in 2 millilitres of BHI broth, these cultures them overnight at 37°C. The following day the cultures were held at 37°C in a waterbath for at least one hour after addition of a further 2 millilitres of BHI broth,this brings them into log-phase growth, and gives a cell density of about 10^9 cells/ millilitre. These cultures were spun down and resuspended in 2 millilitres of fresh BHI broth. 0·1 millilitres from the Hfr culture were withdrawn and mixed with 1·9 millilitres of the recipient in a sterile bijou bottle; this provides an adequate surface for oxygen diffusion. Immediately after mixing, a 0·01 millilitre aliquot was withdrawn and added to 14·99 millilitres of phosphate buffered saline (PBS), and strongly vortexed to halt mating. The dilution (1 into 1500) was

sufficient to prevent the formation of further mating pairs. This served as a zero-time control.

Immediately after the zero-time control was removed, a 0·1 millilitre aliquot from the same mating mix was withdrawn, and added to 50 millilitres of prewarmed BHI broth. This was incubated at 37°C. At appropriate and predetermined intervals 1 millilitre samples from the diluted mating mixture were withdrawn and placed in test-tubes, vortexed to separate mating cells, spun down as before and then resuspended in 3 millilitres of PBS. This preparation or a suitable dilution was plated in duplicate on suitable selective agar plates.

The markers chosen for this mapping experiment were utilisation of D-methionine (Met D$^+$, mapping at 4½ minutes on the E.coli K12 gene map) and threonine utilisation (Thr$^+$, located at 0 minutes on the E.coli K12 gene map). Crosses, with F-2-3-27 as the Hfr were used as a negative control since this strain does not carry the W_k prophage. A D_2-18/3 Hfr (carrying the 808 F plasmid insertion) was used for the timing experiments.

The results obtained in these crosses placed the met D and the thr genes at the correct map positions and showed that the method being used was valid. Unfortunately, when attempts were made to time the entry of the W_k prophage, no useable results were obtained. The selection used to isolate the cells which had received the W_k prophage was the ability of exconjugants lysogenic for the W_k prophage to exclude phage P_1. However, when exconjugants were plated onto a P_1 lawn, no P_1-excluding colonies were found. This proved to be due to a lag in the expression of the restriction system encoded by the W_k prophage. Since this meant that W_k^+ cells could not be selected directly from the mating mix, the time of entry could not be readily determined. Some useful information was gained

however: when exconjugants from both test crosses were examined, it was found that approximately 85 % of the Met D$^+$ exconjugants were W_k^+, regardless of the time at which mating had been interupted. Approximately 65 % of the Thr$^+$ exconjugants were W_k^+, also regardless of the time at which the mating had been interrupted. This implies that the W_k prophage had entered the recipient cell ahead of the two selected markers. This observed linkage must be due to transferral of the prophage by conjugation since any W_k phage free in the mating mix would be unable to adsorb to the recipient strains. It was decided in view of the above, to map the position of the W_k prophage by a different strategy, viz., its relative linkage to known markers.

Mapping the insertion site of the W_k Prophage in E.coli strain W : Relative linkage to adjacent genes.

The work previously described gave strong evidence for the insertion site of the W_k prophage being close to the met D locus. Together with the strong linkage to the pro A,B locus described in Experimental section 2, this implied that the W_k prophage insertion site was close to 5 minutes and probably beyond the met D locus. The Thr$^+$ marker is not as strongly linked to transmission of the W^k prophage as is desirable for linkage studies. A decision was thus taken to derive a strain with a mutation at a locus more favourably placed to enable the exconjugants from a cross to be scored for relative linkage in a 3-factor cross.

The Met D$^-$ phenotype is due to the absence of a permease permitting the uptake of D-methionine. Those cells with the relevant permease are able to take up D-methionine, convert the D-form to the natural isomer,

L-methionine and in this way satisfy a methionine requirement. Methionine auxotrophs (Met$^-$) which lack the permease are unable to grow on media supplemented with D-methionine. A Met$^-$ mutant of E.coli C was chosen as the recipient strain since it was established in the course of this work that this strain is naturally deficient for the D-methionine permease. Thus, any Met$^-$ E.coli C exconjugants from the proposed cross which failed to receive the met D locus are unable to take up the D-methionine.

It was intended that the prophage itself would serve as another marker, being scored for its ability to confer restrictive ability on E.coli C against phages P_1 and Lambda.

The third marker chosen was resistance to phages T_1 and T_5. This phenotype is conferred by mutations at the ton A locus which is located at approximately 3¾ minutes on the standard E.coli K12 gene map [Bachmann 1983].

A Ton A^r mutant of the Met$^-$ E.coli C strain, C-1463, was derived by growing the cells overnight at 37°C in 2 millilitres of T-broth and then adding 0·1 millilitres of a high titre stock of phage T_1 and incubating overnight at 37°C ; this allowed lysis of cells sensitive to the phage. The lysate was then inoculated into 2 millilitres of fresh T-broth and incubated at 37°C until the resistant cells had grown up to a cell density of approximately 10^9 cells/millilitre ; 0·2 millilitres of a stock culture of phage T_5 was added and the mixture incubated overnight at 37°C to allow lysis of cells sensitive to this second phage. The phage lysate was then streaked on BHI agar plates and single colonies selected and tested for genetic markers, in particular Ton A^r. By these successive treatments, it was possible to derive a C-1463 mutant carrying the Ton A^r marker conferring resistance to phages T_1 and T_5. The Ton A^r marker was

derived in the E.coli C strain rather than the E.coli W Hfr since the E.coli W restriction of phage T_1 is so efficient that even very high multiplicities of infection failed to completely lyse all the senstive cells when grown in broth. An interesting observation is that this restriction to the T-odd phages is not observed in strains of E.coli lysogenic for the W-phage other than E.coli W [Jamieson 1971, also this study]. This leads to speculation that there may be further mechanisms of restriction in E.coli W not yet explored.

The Ton A marker was checked by measuring the adsorbance coefficient of phages T_1 and T_5 to putative Ton A^r and Ton A^s cultures of C-1463. K_{ADS} is determined by the following formula :

$$K_{ADS} = 2.3/ (B)t \times \log P_0/P$$

where : P_0 = phage titre at time t = 0

P = phage titre at time t

B = number of bacterial cells per millilitre

K_{ADS} = Velocity constant with dimensions of cubic centimetres/ minute

See figure 8.

[Glover et al 1967, Jamieson 1970].

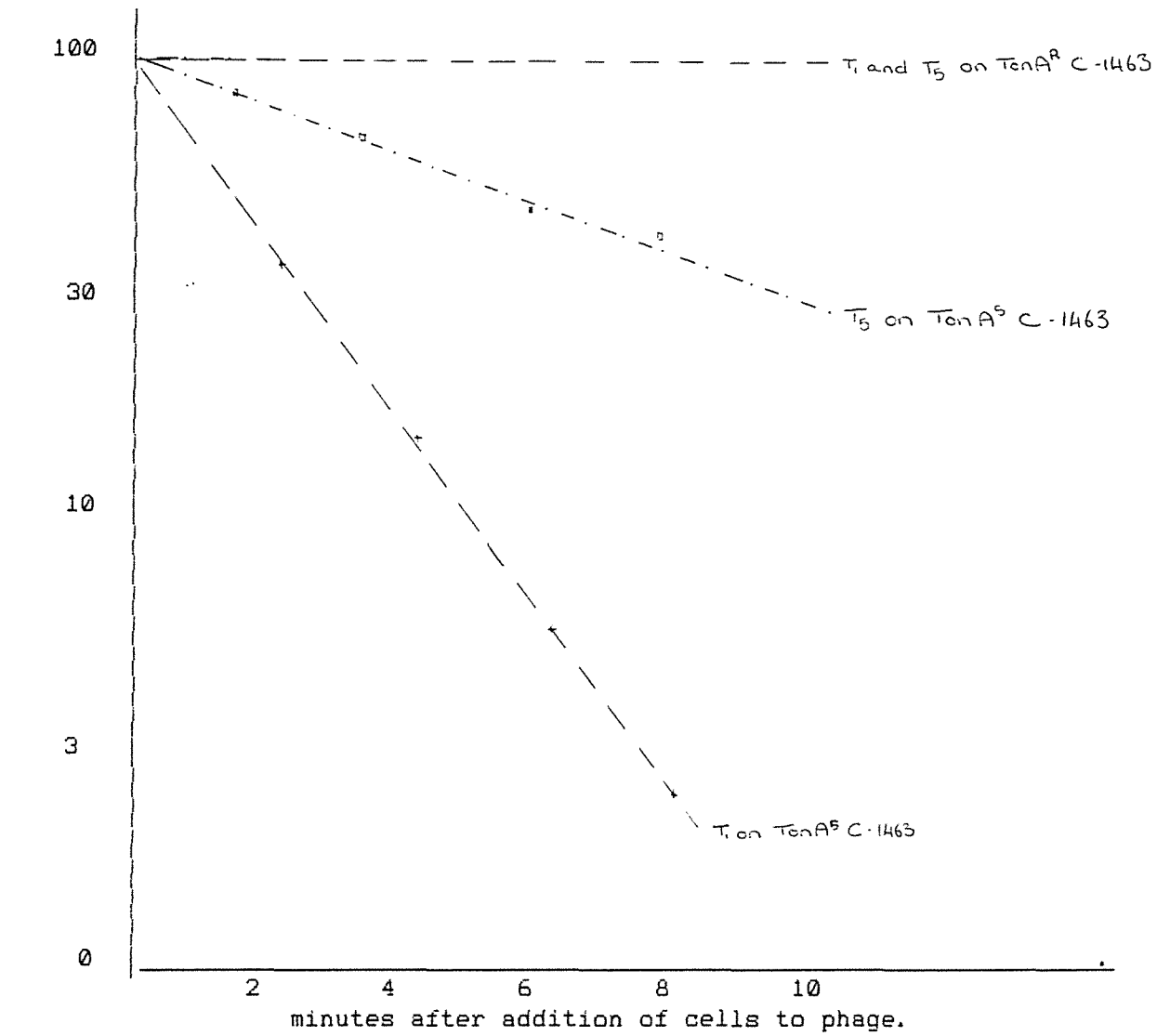


FIGURE 8 : ADSORPTION OF T1 AND T5 TO TON A^R AND TON A^S STRAINS OF C-1463.

2 conjugations were then set up using the D_2-18/3 Hfr and the Ton A^r Met D^- C-1463 to test linkage of the W_k prophage to the 2 markers chosen. Each cross was selected for one of the markers (Met D^- or Ton A^r) and then scored for the transmission of the W_k prophage (restriction of phage P_1 and release of W_k phage). This was done to ascertain the degree of linkage of the markers as a guide for the 3-factor cross

Linkage of Selected Markers to the W_k Prophage.

Selected Phenotype	Number of cells screened for linkage	Number of P_1 resistant colonies:	Linkage to selected marker:
Met D^+	95	79	83 %
Ton A^r	97	75	77·3 %

P_1 restriction can also be expressed in the form of the E.coli W type I restriction system. This restriction system can be differentiated from the W-prophage coded system by the fact that it is less effective and also expresses a modification system capable of protecting phage. The figures quoted above are only for the W-prophage type restriction since the phage used for this test were modified against the hsdW restriction. The tight linkage of both these markers to W_k prophage restriction indicated their suitability for use in a 3-factor cross.

This gave 3 potential markers Ton A^r, Met D^+ and $P_1{}^r$ in the area to which the W_k prophage had been tentatively mapped (see figure 9). The Ton A^r derivative of the C-1463 strain was used as a recipient in a cross with the D_2-18/3 Hfr strain and plated on selective media selecting for Met D^+ exconjugants.

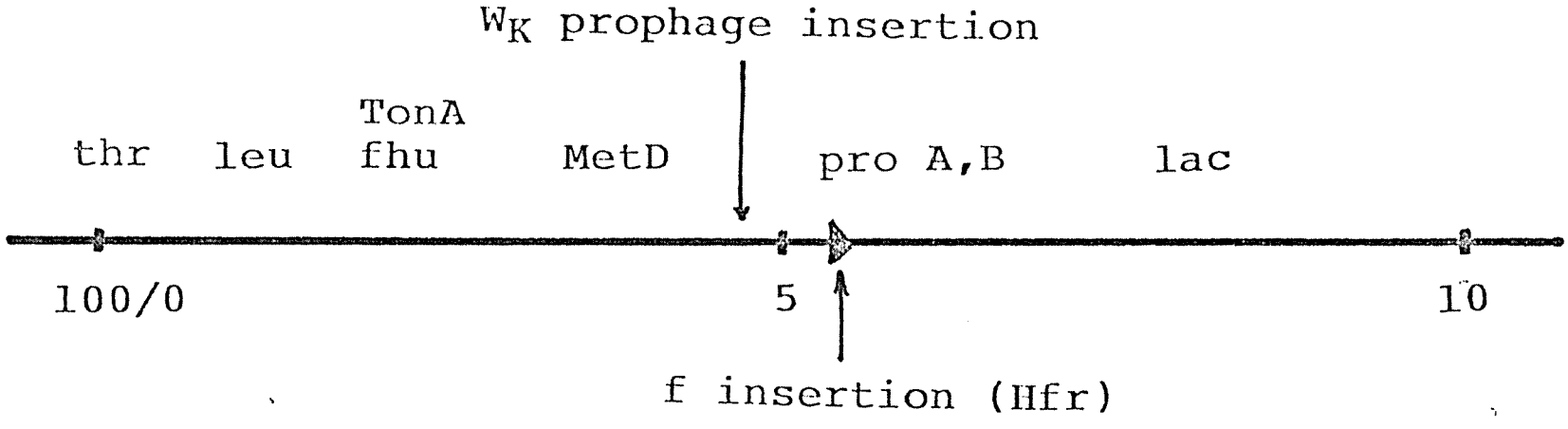


FIGURE 9: GENE ORDER OF MARKERS USED TO MAP THE Wk PROPHAGE.

The exconjugant colonies which grew were picked and single-colony purified. The purified colonies were picked, taking care to take only a small inoculum and inoculated into 0·1 millilitres of BHI broth in a well of a microtitre plate (too heavy an inoculum makes reading of the subsequent test difficult). The cultures in the wells were then incubated at 37°C for one hour to give a light growth.

A Modified Lennox (ML) agar plate was divided into six sections by horizontal lines, and a loopful from each culture streaked across an individual section with a sterile loop. Both microtitre wells and the inoculations on the agar plates were labelled to ensure that cultures could be later identified. The culture streaks were allowed to dry. The high titre phage suspension to be applied, in this case phages T_5 and P_1, were spotted onto opposite ends of the dried streaks, allowed to dry and the plates were then incubated overnight at 37°C.

An area of complete lysis by either phage was interpreted as sensitivity, since the titre of the phage spotted onto the plate was high, while partial lysis, (isolated plaques of P_1) was taken to indicate transferral of the hsdW restriction. No plaques on the streak was taken to mean that the colony exhibited strong W-prophage type restriction to P_1 or resistance to T_5. Controls for the experiment included both parents from the cross ; they behaved as predicted. The results for testing 95 exconjugants are summarised in the table below :

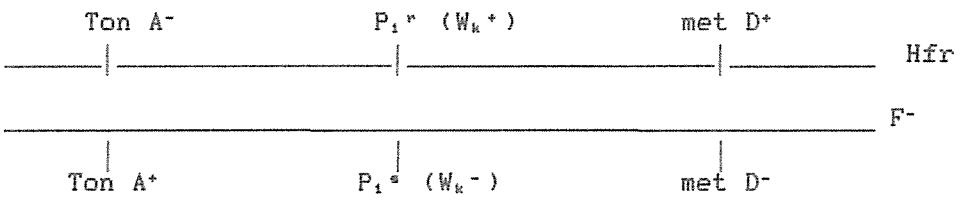
Phenotype of Exconjugant	Number in sample	% in sample
met D^+ P_1^s T_5^s :	14	15
met D^+ P_1^r T_5^s :	61	64
met D^+ P_1^s T_5^r :	1	1
met D^+ P_1^r T_5^r :	19	20

An analysis of these results is provided in figure 10. The phenotype with the lowest frequency was predicted to be the one requiring the statistically least likely combination of crossover events, that is, 2 crossover events within the confined area of interest. The remaining, more frequent phenotypes can be generated by a single crossover in the region of interest and a second crossover outside the region of interest. These are described in figure 10. By these parameters the gene order, reading clockwise on the standard E.coli K12 gene map, is ton A, met D, prophage W_k, the insertion site for Hfr 808 and pro A,B. The approximate map positions* are :

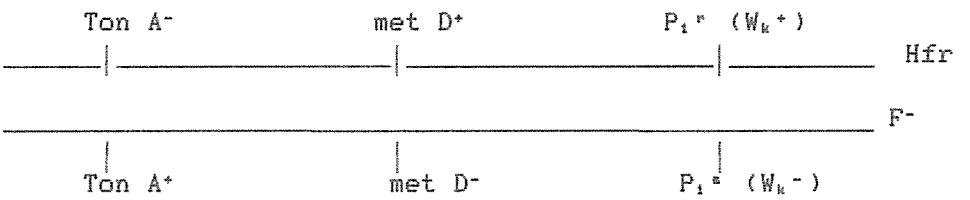
ton A	3½ minutes.
met D	4½ minutes.
W_k	5 minutes.
Hfr 808	5½ minutes.
pro A,B	6 minutes.

*After Bachmann, 1983.

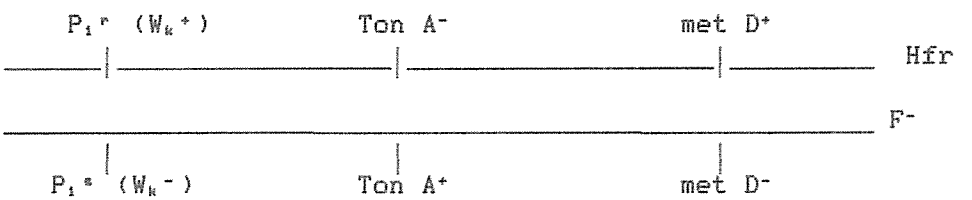
These figures show the 3 possible arrangements of the genes used in the 3-factor cross. The least common class will be the one which requires a double cross-over within the bounds of the markers shown to generate it. Thus by finding the least common class of exconjugant in such a cross, the correct gene order can be deduced.



Possible exconjugants : met D⁺ P₁ˢ T₅ˢ.....Least Common Class.
met D⁺ P₁ˢ T₅ʳ
met D⁺ P₁ʳ T₅ˢ
met D⁺ P₁ʳ T₅ʳ



Possible exconjugants : met D⁺ P₁ˢ T₅ˢ
met D⁺ P₁ˢ T₅ʳ.....Least Common Class.
met D⁺ P₁ʳ T₅ˢ
met D⁺ P₁ʳ T₅ʳ



Possible exconjugants : met D⁺ P₁ˢ T₅ˢ
met D⁺ P₁ˢ T₅ʳ
met D⁺ P₁ʳ T₅ˢ
met D⁺ P₁ʳ T₅ʳ.....Least Common Class.

FIGURE 10 : PROBABILITIES OF GENERATING DIFFERENT PHENOTYPES IN EXCONJUGANTS FROM A THREE-FACTOR CROSS.

EXPERIMENTAL SECTION 4

ANALYSIS OF W-PHAGE AND PHAGE Mu DNA.

As was outlined in the Historical Review, there are a significant number of similarities between the W_o and W_k phages on the one hand and phage Mu on the other. All three are temperate phages, there are striking parallels in the patterns of propagation of the phages, they are similar morphologically and, interestingly, can induce mutations by insertion into sites which are distributed at random along the host cell chromosome (See also figure 3). In view of these similarities, attention has been devoted to clarifying the relationship of these phages [Rice 1980, Moody 1983]. These studies revealed minor differences between Mu and the W-phages but have failed to clarify the relationship between these phages. It was therefore decided that an examination of these phages at the molecular level might better provide an indication of the degree of their relatedness.

DNA from the three phages (Mu, W_o and W_k) was examined for restriction patterns and DNA homology. To this end, phage DNA was extracted by the following method ;

Preparation of Bulk Phage Stocks From Single-plaque Isolates.

1) Phage Mu

The Mu phage strains used in these experiments are temperature-sensitive derivatives of the temperate phage Mu-1 obtained as lysogens of E.coli K12 from Dr Martha Howe : Mu cts 61, which is Gin$^+$, and Mu cts 62,

which is Gin$^-$. The Gin$^-$ strain was chosen as the most suitable vehicle for restriction endonuclease analysis for several reasons. The first, and most important reason is that this phage is defective for the G-inversion (gin) gene. A consequence is that, if the phage is grown up by induction, no confusion will arise during restriction endonuclease analyses due to inversion of the G-region. The temperature-sensitive mutant is also particularly suitable for deriving bulk high titre lysates, as it is considerably easier to derive large numbers of phage by induction than by lytic growth from a single plaque. Finally, use of the Mu cts 62 strain is attractive in that it has been widely used for genetic analysis in the past and there is a considerable body of literature dealing with this particular Mu phage derivative.

Since the phage was to be prepared by induction rather than lytic growth, a single colony of the E.coli K12 lysogen was the starting point rather than a single plaque. The culture of Mu cts 62 was streaked for single colonies on a BHI agar plate and incubated overnight at 30°C. The following day a single colony was picked and inoculated into 100 millilitres of T-broth which was further incubated overnight at 30°C with shaking. The following day this culture was used to inoculate 2 x 500 millilitres of T-broth, prewarmed to 30°C, to give an optical density of 0·05 at a wavelength of 600 nanometres (OD_{600}). The inoculant cultures were then incubated for 3 - 4 hours at 30°C with shaking, until the OD_{600} of the culture reached 0·5. Immediately this stage was reached, the cultures were induced by incubating for 20 - 25 minutes in a waterbath at 45°C with shaking; it is essential that the entire volume reaches 45°C as quickly as possible. It is for this reason that the largest volume to which this method can conveniently be adapted is 500 millilitres and is

the reason why the 1 litre culture was split between two flasks. After heat induction, the flasks were incubated at 37 - 38°C for a further 2½ - 5 hours with vigorous shaking. Within this period, lysis of the induced culture becomes apparent, with a marked decrease in turbidity and the formation of large clumps of cellular debris. At this stage, 5 millilitres of analytical grade chloroform were added to each culture and incubation at 37°C continued for a further 30 minutes to complete the lytic process and kill off any cells which have survived. The induced culture was centrifuged at 10,000 g (9,500 rpm in a Sorvall GSA rotor) for 10 minutes to remove gross cellular debris and then titrated. This process should yield 1 litre of phage suspension with a titre of approximately 10^{10} plaque-forming units per millilitre (PFU/ml). The phage purification and DNA extraction procedure applied, was the same as that for the W-phage and is described below.

2) The W-phage.

The W_o and W_k phage were obtained by lytic propagation. The host used to propagate the W_o phage was the restrictionless E.coli C strain 518C, while the host used for the W_k phage was the restrictionless E.coli K12 mutant PB1395; this host strain still possesses modifying activity. The initial phage used in this procedure were taken from a single plaque. Modified Lennox (ML) agar was used for the basal layer; and Modified Lennox soft agar was used for the overlays. A single plaque was picked and inoculated into 0·3 millilitres of exponential host cells in BHI broth, vortexed, allowed to adsorb for 15 minutes and then spotted onto a lawn of the host cells in ML agar. After overnight incubation at 37°C, a small area of confluent lysis was obtained. The area of confluent lysis

was removed with a sterile pasteur pipette and added to 0·5 millilitres of exponential host cells (approximately 10^9 cells/ml) in BHI broth, vortexed, and allowed to adsorb for 15 minutes. 0·1 millilitres of this mixture was added to each of 5 ML soft agar overlay plates after adsorption, along with the tetrazolium solution and seed bacteria, then poured onto a ML basal plate. After overnight incubation at 37°C, these plates displayed confluent lysis. The phage was harvested from these plates by adding 2·0 millilitres of T-broth to each plate and the soft agar and broth scraped off into a sterile test-tube. As this operation involved the exposure of the plate surface, it was performed in a laminar-flow cabinet. 6 - 7 drops of analytical grade chloroform were added to each test-tube and the contents vortexed to break up the agar and release the phage. After vortexing the mixture was centrifuged at approximately 4000 g to pellet the agar. The supernatant, with approximately 10^{10} PFU/ml, was poured off and titrated. Storage of the phage prepared by either method is the same as that already detailed in the general methods section but, since there may be a drop in the titre of the phage, the full-plate lysis step was normally done immediately before it was required to grow up to the full 1 litre volume.

The 1 litre high titre lysates were prepared by adding the "confluent lysis" preparation of phage to a concentrated culture of host cells in the log-phase of growth. The host culture was prepared by inoculating 30 millilitres of T-broth with the appropriate host and growing it up overnight at 37°C. These 30 millilitres were used to inoculate 650 millilitres of T-broth which was then incubated at 37°C with shaking for 3½ to 5 hours, until the culture reached a cell density of about 5 x 10^8 cells per millilitre. The culture was immediately chilled in crushed ice

and concentrated by centrifuging at 4000 g for 10 minutes at 4°C. The cells were resuspended in 65 millilitres of of chilled T-broth to provide the concentrated log-phase cells. These were held in ice until they were required.

2·5 millilitres of the suspension of log phase cells were added to the 10 millilitres of concentrated phage lysate and the mixture held at 37°C for 10 minutes with aeration to allow adsorption of the phage to the cells. 38 millilitres of T-broth, prewarmed to 37°C, was added and the culture incubated at 37°C with aeration until lysis occurred, usually in 1 - 1½ hours.

Following lysis, 12·5 millilitres of concentrated host cell culture was added to the 50 millilitres of phage lysate and the culture incubated at 37°C with aeration to allow adsorption as before. After preadsorption, 190 millilitres of prewarmed T-broth was added and the mixture incubated to full lysis as before. If full lysis is not obtained and the mixture appears turbid, this may be due to the accumulation of resistant cells. In this case, add 2·5 millilitres of analytical grade chloroform and incubate at 37°C for 20 minutes; the chloroform can be removed by aeration and the lytic process continued.

Once the culture had lysed, 50 millilitres of the concentrated log-phase culture was added to the lysate and held as before to allow preadsorption before 700 millilitres of prewarmed T-broth was added and aerated at 37°C until the culture cleared. This final lytic propagation may take 2½ to 4 hours. The litre of lysate obtained was incubated at 37°C for 30 minutes with shaking, after the addition of 10 millilitres of analytical grade chloroform. This lysate was centrifuged at 10,000 g for 10 minutes to remove cellular debris (about 9,500 rpm in a Sorvall GSA

rotor) and stored over chloroform (at 1 % concentration, v/v). Typically the above method yields a lysate with approximately 10^{10} PFU/ml.

Isolation and Purification of Phage DNA.

1) Purification of Phage particles.

The high-titre bulk phage lysate was brought up to room temperature (if this procedure carries directly on from preparation of the lysate, it may simply be left on the workbench overnight) and then crude RNAase and crude DNAase added to a final concentration of 1 microgram per millilitre (a milligram into the 1 litre bulk lysate). The lysate was then incubated at room temperature for 30 minutes to allow the enzymes to degrade any free cellular nucleic acids.

At this point, NaCl was added to the lysate to a final concentration of 0·5 moles per litre (29·2 grams/litre) and the lysate held on ice for one hour to precipitate the remaining cellular debris. Once precipitated, the cellular debris was removed by centrifuging the lysate at 11,000 g for 10 minutes at 4°C (10,500 rpm in a Sorvall GSA rotor). The supernatant was carefully decanted, taking care not to disturb any of the precipitated material. This supernatant served in the further purification of the phage.

The phage were collected by precipitation with Polyethyleneglycol (PEG) 6000; the PEG was added to a final concentration of 10 % weight to volume (100 grams/litre) and dissolved with a magnetic stirrer. This preparation was held for 2 hours on ice for the phage to clump and precipitate. The precipitated phage were then pelleted by centrifuging at 14,000 g (12,000 rpm in a Sorvall GSA rotor) for 15 minutes at 4°C. The supernatant was then discarded (a sample from the supernatant was

titrated to monitor phage loss). It is important to remember that phage activity can be greatly decreased by the PEG treatment.

The centrifuge bottles were stood inverted, in a tilted position, for 10 - 15 minutes to allow fluid to drain from the walls and the pelleted phage, any remaining fluid that has accumulated around the neck of the centrifuge bottle was removed with a clean pipette.

The pelleted phage was resuspended in 10 millilitres of Mu-buffer as gently as possible. The walls of the centrifuge bottle were washed when resuspending the phage. The collected phage sample may be titrated, but the activity of the phage is so greatly reduced that it is better to wait until after the removal of the PEG.

The PEG was removed by mixing an equal volume of chloroform with the resuspended phage and vortexing thoroughly for at least 30 to 60 seconds. Then the mixture was centrifuged at 1600 g (4500 rpm in a Sorvall SS34 rotor) for 15 minutes at 4°C. After centrifugation, the upper (aqueous) phase should be clear. If it was at all cloudy, the aqueous phase was removed and extracted again with chloroform. An indication (though not necessarily conclusive) of a good phage yield is a thick white sludge of PEG at the interface of the chloroform and aqueous phases. The upper phase contained the cleaned phage, and was titrated, but the same caution regarding reduced activity applies to titres at this stage as formerly.

The phage were concentrated by centrifugation at about 30,000 g (20,000 rpm in a Sorvall SS34 rotor) at 4°C for 3½ to 4 hours. The pellet of phage, after this treatment, was translucent, pale brown and glassy in appearance. The supernatant was removed and titrated before discarding. 2 millilitres of Mu-buffer was added to the pelleted phage and the pellet left overnight at 4°C (preferably with gentle shaking) to resuspend the

phage. The following day, any phage still pelleted was resuspended by gently taking the fluid up and down in a wide bore pipette. A sample was titrated at this stage; the titre was typically about 10^{13} plaque-forming units per millilitre (PFU/ml). However, phage activity was definately reduced.

2) Extraction of Phage DNA.

To the concentrated phage suspension (in 2 millilitres of buffer), sufficient EDTA was added to give a final concentration of 20 millimoles/litre (normally 0·22 millilitres of a stock 0·2 molar stock solution into 2 millilitres) and sufficient SDS to give a concentration of 0·5 % (0·11 millilitres of stock 10 % solution into 2 millilitres). Also 0·5 milligrams of Pronase was added per millilitre of suspension.

CAUTION!! Pronase should always be purified of any residual DNAase activity by autodigestion in milli-Q or distilled water for at least 2 hours at 37°C. If Protienase K is used instead, no pretreatment is necessary unless prior contamination is suspected.

The phage suspension mixture was briefly vortexed and then incubated at 37°C for 1 hour to allow the phage particles to lyse, with the release of the phage DNA. Beyond this stage, extreme caution was exercised to ensure that bare skin did not come into contact with any working surface which may later have contact with the DNA preparation. This included pipette tips, mouths of test-tubes, dialysis tubing and the like. The reason for this is that skin exudes enzymes with DNAase and RNAase activities and a single mistake in the later phases of the DNA preparation can destroy the preparation.

Once the phage particles were lysed, an equal volume of equilibrated phenol was added to the mixture and the contents mixed thoroughly but gently by shaking. Vortexing is not suitable. The two phases were separated by centrifugation at 1,600 g for 15 minutes at room temperature. A Sorvall type A benchtop centrifuge is ideal for this. The upper, aqueous phase was clear, with a white layer of separated protein at the interface of the buffer and the phenol. If the upper phase had a cloudy appearance, it was necessary to re-spin the preparation. After separation of the phases, the aqueous phase was removed with a wide-bore pipette. Care should be taken to dislodge as little of the phenol and protein as possible. This aqueous phase was mixed with fresh phenol and the entire extraction process repeated at least twice, until no more protein was precipitated.

The aqueous phase was then washed by mixing it with an equal volume of 50/50 equilibrated phenol/ chloroform + isoamyl alcohol and then separating out the aqueous phase by centrifugation as previously outlined. The final wash was performed by mixing the aqueous phase with an equal volume of chloroform + isoamyl alcohol, mixing and recovering the aqueous phase by centrifugation as in the preceding steps.

The cleaned DNA preparation (usually referred to as phenol-extracted) was then transferred to a length of dialysis tubing with sufficient headroom to allow at least 50 % expansion and sealed with clips; it was then dialysed overnight at 4°C in 4 litres of TE buffer with gentle stirring.

The following day, the DNA solution was removed to a clean, sterile test-tube to which had been added 3 molar sodium acetate, to an ammount 1/10th of the final volume of the DNA preparation and, in addition, 2

times the volume of the DNA preparation of cold (-20°C) 95 % ethanol. This mixture was held for 1 to 2 hours at -20°C (or overnight if the DNA concentration is low); the DNA precipitated as a stringy white mass floating in the ethanol.

The DNA was brought down by spinning at 12,000 g for 15 minutes at 4°C; a lower speed is feasible if the DNA pellet is large. The ethanol was simply poured off and the tube drained well on clean absorbant paper. When the tube was dry, the DNA was simply dissolved in the desired ammount of TE buffer. It was found convenient to hold this DNA as a stock and take a small sample to be diluted appropriately as a working stock.

Restriction Endonuclease Analysee of Phage DNA.

After extraction and purification, the DNA was stored at 4°C in 10/1 TE buffer and was found to be stable under these conditions. The concentration of the DNA prepared by the above method was readily ascertained by finding the absorbance of the DNA at 260 nm and 280 nm in a spectrophotometer. The ratio of the absorbance of the DNA at 260 over the absorbance at 280 should be between 1·7 and 2·0. If the ratio is lower than this, the DNA contains contaminating material, probably protein, and should be extracted with phenol and/or treated with protease. An optical density of 1·0 at 260 nanometers corresponds to a concentration of 50 micrograms of DNA per millilitre for clean DNA.

Using this method, the DNA prepared for this experiment had a concentration of 800 - 900 micrograms per millilitre and for experimental purposes was diluted by a factor of 10, so that 2 microlitres contained approximately 1.5 micrograms of DNA. It is desirable that the quantity of DNA in the restriction digest does not exceed 2 micrograms, since this

will overload the restricting capacity of the enzyme leading to incomplete digestion and consequent smearing of the gel. If extra enzyme is loaded into the digest, the concentration of the storage buffer (usually glycerol-based) of the enzyme in the digest mixture will increase, and upset the delicate ionic balance upon which the effectiveness of the enzyme is dependant. This may lead to incomplete digestion, or non-specific activity by the enzyme, decreasing the value of the gel.

A large number of enzymes were used to cut the W-phage and Mu phage DNA. These were chosen to provide fragments which could be matched against published maps of phage Mu [Bukhari et al. 1977] These are summarised in figure 11. It was decided that it was beyond the scope of this project to attempt to construct a restriction map for the two W-phage, but all the evidence collected to date suggests that they are extremely similar at the genomic level, if not identical.

The digestion of the DNA with various enzymes followed a similar pattern, but the volumes of the reagents used varied with the salt tolerance of the enzyme in question. The digestion mix was made up of a number of "cocktails" which were addded together in varying proportions. These are detailed below :

x 10 Hae III buffer

60 mM Tris pH 7·6.....1·2 ml 1 M Tris stock

100 mM $MgCl_2$.....2·0 ml 1 M $MgCl_2$ stock

100 mM Mercaptoethanol.....142 µl ß-Mercaptoethanol

Distilled water to 20 ml.

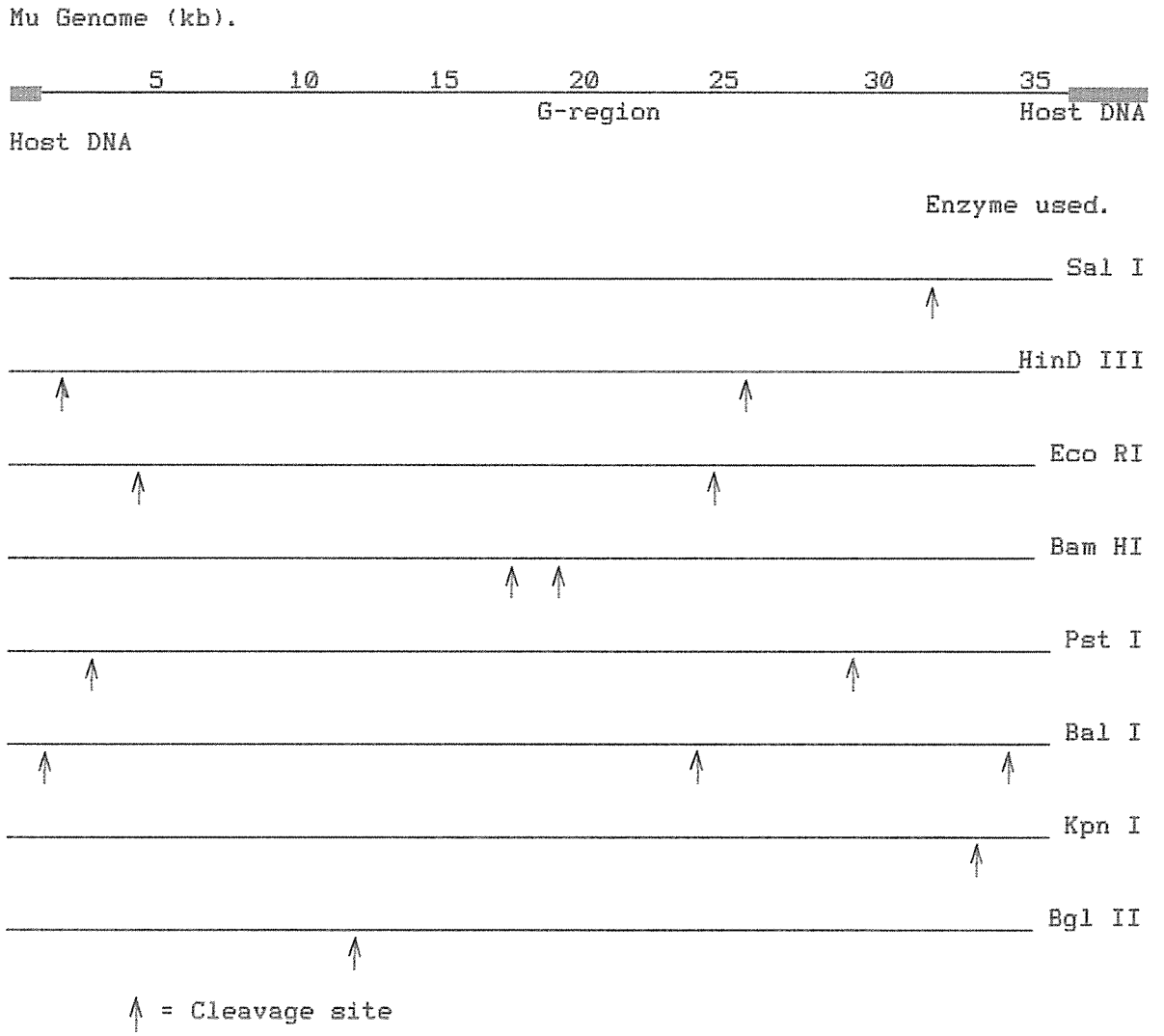


FIGURE 11 : CLEAVAGE SITES OF RESTRICTION ENDONUCLEASES USED TO EXAMINE THE W-PHAGES AND PHAGE Mu. (After Bukhari et al. 1977).

Dye mix

20 % Sucrose.....4·0 g

5 mM Na_2EDTA pH 8.....0·5 ml 200 mM EDTA stock

1 % SDS.....0·2 g

0·2 % bromophenol blue.....40 mg

Distilled water to a final volume of 20 ml

Restriction digests were prepared by the following method : Approximately 1 microgram (µg) of DNA (2 µl of stock working solution in 10/1 TE buffer) was added to 2·5 µl of x 10 Hae III buffer, the appropriate ammount of NaCl (see below), 3 units of enzyme, and sufficient distilled water to make the final volume up to 25 µl, in a 1·5 millilitre Eppendorf tube. The activity of the enzyme varied depending on the manufacturer and is displayed as x units/ml. The ammount of 1 M NaCl which was added to a 25 µl reaction mix is detailed below :

Enzyme :	Volume of 1 M NaCl added to 25 µl :
Kpn I	0·15 µl
Pst I, Hind III	1·25 µl
Eco RI	2·50 µl
Bgl II, Bam HI, Sal I	3·75 µl

There are a few enzymes which display lowered specificity if the reaction conditions are not exactly right; these enzymes gave best results if used in a "purpose-made" buffer (requirements are usually listed in the manufacturers catalogue). Enzymes in this category which were used include Bal I and Pst I. Finally, some manufacturers now produce low, medium, and high salt buffers in a x 10 concentrate. These

may be used (2·5 µl) for the designated enzymes in place of NaCl and Hae III buffer, and the water adjusted to make up the extra volume.

The reaction digest was vortexed well and spun briefly in an Eppendorf centrifuge to remove any droplets which might be clinging to the wall of the tube. The tube was then incubated at 37°C for 1 - 2+ hours, at which point the reaction was halted by the addition of 5 µl of SDS dye and the tube spun briefly in an Eppendorf centrifuge to bring down any drops which had condensed in the upper parts of the tube. It should be noted that some enzymes do not have maximum activity at 37°C. The exact conditions required for optimum activity are given in the manufacturers catalogue, and were always checked before setting up a digest.

The reaction mix was then transferred into a slot on an agarose gel and allowed to run for the appropriate length of time. The current to which the gel was subjected, the density of the gel and the length of the run depend on the purpose of each digest and are therefore discussed below as appropriate.

Restriction Patterns of the DNA from the W-phages and Phage Mu.

The following pages contain records of restriction gels done as described above, together with descriptions of the reaction conditions and any relevant comments.

Plate #1. 2 µl each of Mu, W_h, and W_e DNA (reading from left to right) digested for 2 hours at 37°C with Hind III, then 2 further hours with EcoRI. Run on a 1% gel at 45 volts for 10 hours and visualised by soaking the gel in 0·5 % Ethidium bromide for 15 minutes before photographing under UV light with a MP.4 Polaroid Land Camera. Note that although banding of the DNA from the 2 W-phages is apparently identical, it differs from the banding produced from the Mu DNA.

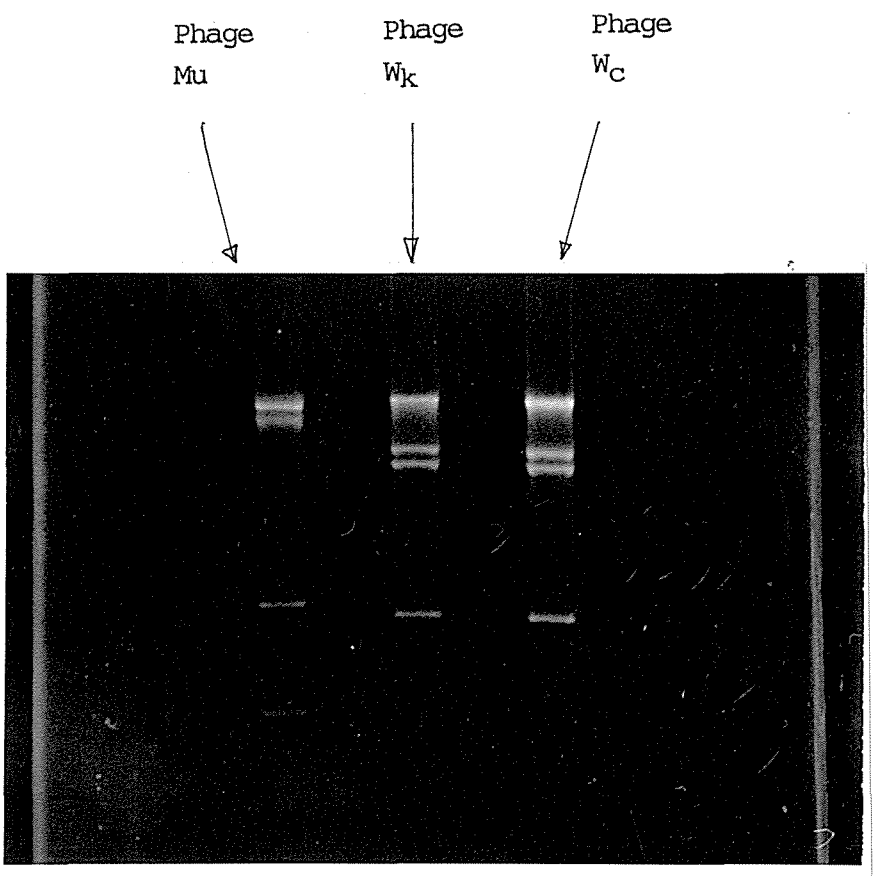
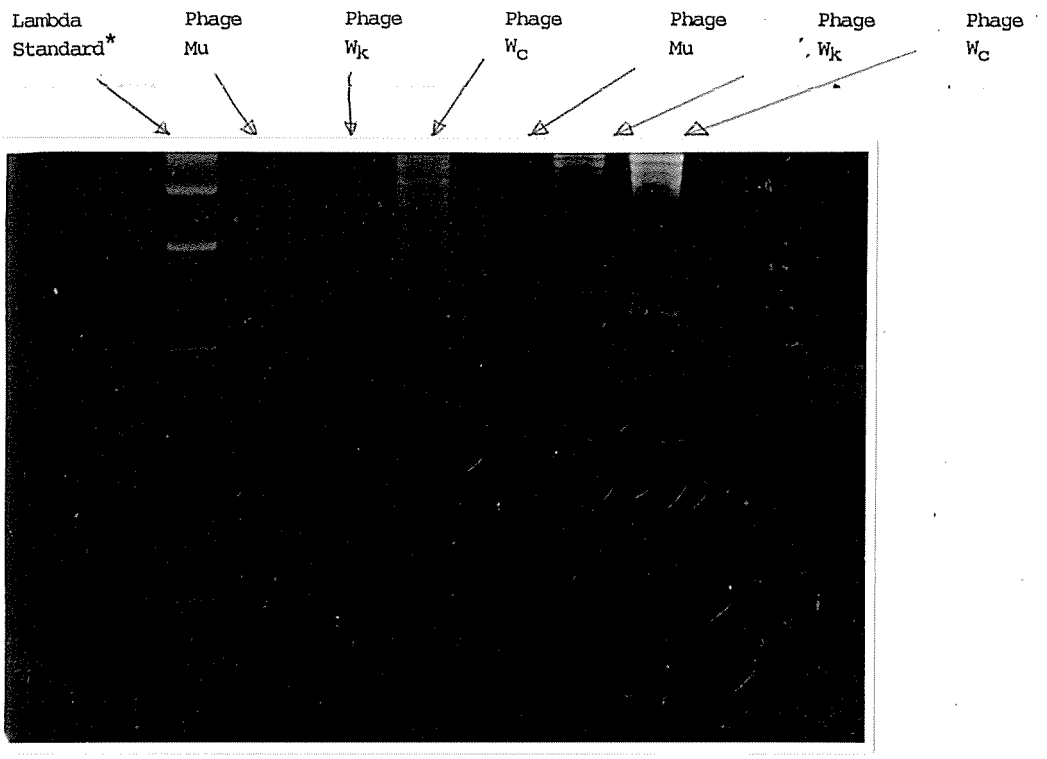
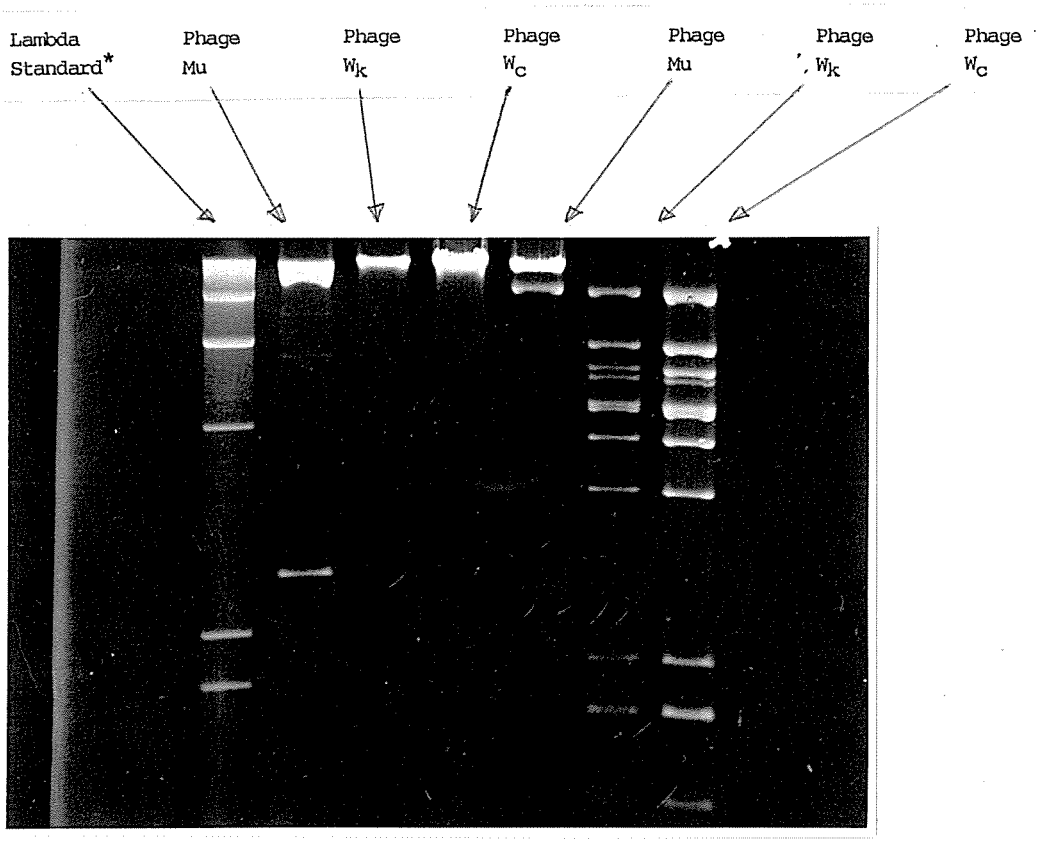

Phage
Mu
Phage
W_k
Phage
W_c

Plate #2. On the left, 3 µl each of Mu, W_k, and W_c DNA (reading from left to right) digested for 2 hours at 37°C with Kpn I, and on the right, 3 µl of DNA in the same order digested for 2 hours with Sal I. Run on a 0·7% gel at 50 volts for 10 hours and visualised by soaking the gel in 0·5 % Ethidium bromide for 15 minutes before photographing under UV light with a MP.4 Polaroid Land Camera. The Kpn I slots are of little use for identification, since some variation in the reaction mix has caused it to display non-specific cutting activity. It is worth noting the prominent band visible in the Mu digest just below the second visible band of the lambda standard. This band has been observed in other digests of Mu where the specificity of this enzyme has been impaired. It is not visible in the W-phage digests. Some incomplete cutting is also visible in the Sal I digest. The upper 3 bands of W-phage DNA shown in the plate correspond to DNA which has lost: (i)the smaller of the 2 DNA bands visualised in the photo, (ii) the larger of the 2 DNA bands, and (iii) both of these DNA fragments (from top to bottom). Again the 2 W-phage samples appear identical, and different from the pattern observed in Mu.



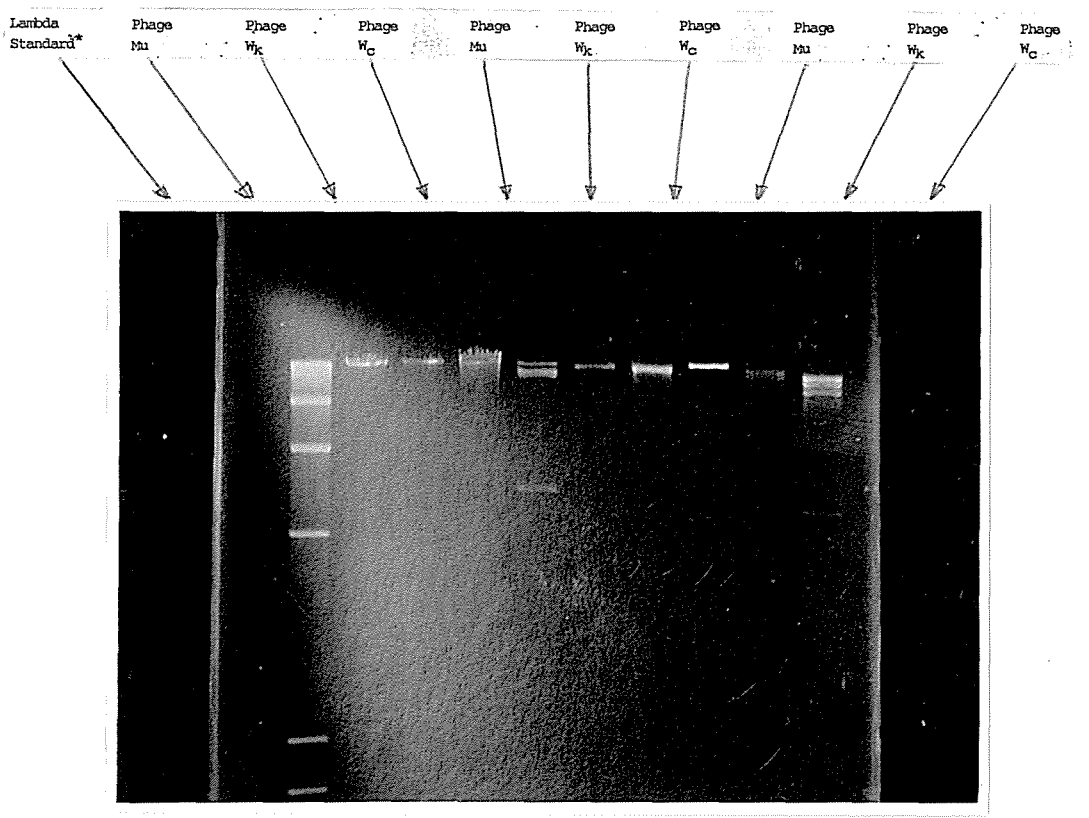
*Cut with Hind III

Plate #3. On the left, 5 µl each of Mu, W_k, and W_c DNA (reading from left to right) digested for 2 hours at 37°C with Bam HI and on the right, 5 µl of DNA in the same order digested for 2 hours with Bgl II. Run on a 1 % gel at 45 volts for 10 hours and visualised by soaking the gel in 0·5 % Ethidium bromide for 15 minutes before photographing under UV light with a MP.4 Polaroid Land Camera. In this plate the pattern previously observed of marked similarity between the W-phage and major differences between W-phage and Mu-phage DNA is accentuated. The large number of cleavage sites revealed in the W-phage by treatment with Bgl II is likely to show up any major differences between W_c and W_k, but none are apparent. This is further support for the proposal that the phages W_c and W_k are the same. This restriction pattern was readily repeated and all the evidence is that the large number of fragments are not due to non-specific cutting. The fainter bands visible in the Bam HI digest of the Mu DNA (flanking the third band of the lambda standard) are produced by non-specific cutting of the DNA by the Bam HI enzyme (not unusual for this particular enzyme). Some incomplete digestion of the Mu DNA is also visible in the same lane.



*Cut with Hind III

Plate #4. On the left, 2 µl each of undigested Mu, W_k, and W_o DNA (reading from left to right). In the centre, 2 µl each of Mu, W_k, and W_o DNA (reading from left to right) digested for 2 hours at 37°C with EcoRI, and on the right is 2 µl of DNA in the same order digested for 2 hours with Sal I. Run on a 1 % gel at 45 volts for 10 hours and visualised by soaking the gel in 0·5 % Ethidium bromide for 15 minutes before photographing under UV light with a MP.4 Polaroid Land Camera. The smearing effect of the uncut phage DNA is due to overloading of the gel. However, it is possible to see that the front of the W-page DNA preparations seem to be running behind that of the Mu DNA, implying that the W-phage DNA is of a larger molecular weight than the Mu DNA. As previously noted, there are major differences between W-phage and Mu DNA when cut with either enzyme. Unfortunately the plate is somewhat marred by over-exposure, but its major importance is as a visual check for a probe with labelled W_o DNA. (See Plate #5.)



*Cut with Hind III

The Preparation of Radioactively Labelled DNA to be Used as Probes.

The segment of DNA which was to be used as a probe (and a sample of lambda DNA since a labelled lambda standard was not available) was diluted so that 1 - 2 µg of the DNA would be included in a 25 µl mixture to be digested with Hae III for 30 minutes at 37°C. 4 µl of a stock containing 25 mg/ml of random primers was added to each tube and the reaction mix boiled for 2 minutes. The reaction tubes should have their lids opened a fraction before boiling to allow the pressure to equalise. The tubes were removed from the boiling water and immediately placed into ice and allowed to cool for 3 - 5 minutes. The following reagents were then added (in the order given) to the tubes which already contained the Hae III digest and the random primers :

2·5 µl distilled water

1·5 µl Hae III buffer

1·0 µl 20 mM dGTP

1·0 µl 20 mM dATP

1·0 µl 20 mM dTTP

3·0 µl [^{32}p] dCTP (3000 Ci/mM)

2·0 µl Klenow fragment (DNA Polymerase I lacking exonuclease activity)

total : 40 µl

This mixture was incubated for 30 minutes at 37°C, following which 2 µl of 250 mM Na_2EDTA was added to chelate any Mg^{2+}. The reaction mix was extracted with equilibrated phenol : chloroform using 50 µl of each, and spun in an Eppendorf centrifuge for 2 minutes to separate the aqueous phase. The aqueous phase was removed with a wide-bore pipette and the leftover phenol : chloroform mixture was extracted with a further 50 µl of water. The phases of this second extraction were separated by

centrifugation in an Eppendorf as before. Both first and second samples of the aqueous phase were checked for radioactivity after removal of the phenol : chloroform phase; almost all of the radioactivity should be in these aqueous samples. If not, extract the phenol : chloroform mixture with a further 50 µl of water. The aqueous phases were then pooled and loaded onto a column filled with G-50 (fine) Sephadex™ beads equilibrated in 10/1/100 TES. The column effluent was collected in 3 drop fractions. The fractions which contained the bulk of the labelled DNA were ascertained with a hand Geiger counter. The fractions continued to be collected until the radioactivity was seen to have passed out of the column as detected with the hand counter. This procedure produced 2 peaks of radioactivity. The first, which was also the largest, represented the labelled DNA, the second was the unincorporated label. The 2 - 4 fractions containing the first peak were pooled and then ethanol precipitated in the usual manner. The ethanol was checked to ensure that the majority of the counts had been precipitated. Depending on the activity of the probe it was then resuspended in an appropriate volume of distilled water. As a rough guide, DNA with counts around 2000 cps was resuspended in 50 µl of water ; this yielded 5 probes. 1 µl should also be added to 10 µl of water in a clean scintillation vial and counted in a scintillation counter to give a more accurate estimate of the level of radioactivity of the label.

Drying Gels onto Nitrocellulose Paper for Probing.

Once the gel had been run to separate the DNA fragments following restriction endonuclease digestion and photographed to provide a record of the position of the fragments, it was probed with radioactively

labelled DNA to determine the presence or absence of specific DNA sequences. To do this, the gel was dried onto nitrocellulose filter paper. Those gels which it was planned would be probed included a sample of the DNA from which the probe was prepared. This served as a positive control and also allowed the degree of hybridisation to be estimated.

The gels were first trimmed so that areas not containing DNA could be discarded and the gels were marked so that their orientation could be determined. The standard method was to cut a large nick in the top left corner. The gel was then washed in a denaturing solution (0·5 M NaOH + 0·5 M NaCl) by shaking it gently in the solution for 30 minutes. Then washed by 2 x 15 minute shakings in a neutraliser solution (0·5 M Tris pH 7·4 + 2·0 M NaOH). This was followed by 2 x 2 minute shakings in 2 x SSC. The washed gel was blotted dry with filter paper and placed on a piece of nitrocellulose filter paper cut so that it was about 2 centimetres larger than the gel in every dimension. This was then placed in the center of a gel-drying block (filter paper on the bottom) and covered with a piece of Glad-wrap that overlaps each edge by 4 - 5 centimetres. The gel dryer was turned on with the maximum water-vacuum obtainable and the gel dried down at 80°C, for 2 hours. When turning off the gel drier, it is important to ensure that the seal is broken first; otherwise the vacuum will suck water back onto the gel. Once dried, the gel was wrapped in Glad-wrap. These preparations keep for a considerable period.

Probing Dried Gels for DNA Homology.

There are a number of treatments for probing dried gels. The one chosen for use in this work was to prehybridise (this reduces non-

specific hybridisation) by soaking in Denhardt's solution at 65°C for 3 hours.

The gel was sealed into a small plastic "bag" made with a heat sealer, and sufficient Denhardt's added to wet the filter paper thoroughly. As much air as possible was squeezed out of the bag before the final side was sealed. The bag was then manipulated gently for several minutes to ensure that the Denhardts was able to penetrate every part of the filter; then the bag was laid in a 65°C waterbath beneath a sheet of glass to complete the prehybridisation.

After this, the bag was cut open at one corner and the majority of the Denhardt's solution drained out. Enough was retained to just wet the whole gel, about ½ a millilitre. Enough of the probe (in 2 x SSC) to contain about 10^4 counts per minute (cpm) was added to the bag, which was then sealed again. The bag was then incubated overnight at 65°C with shaking, as before.

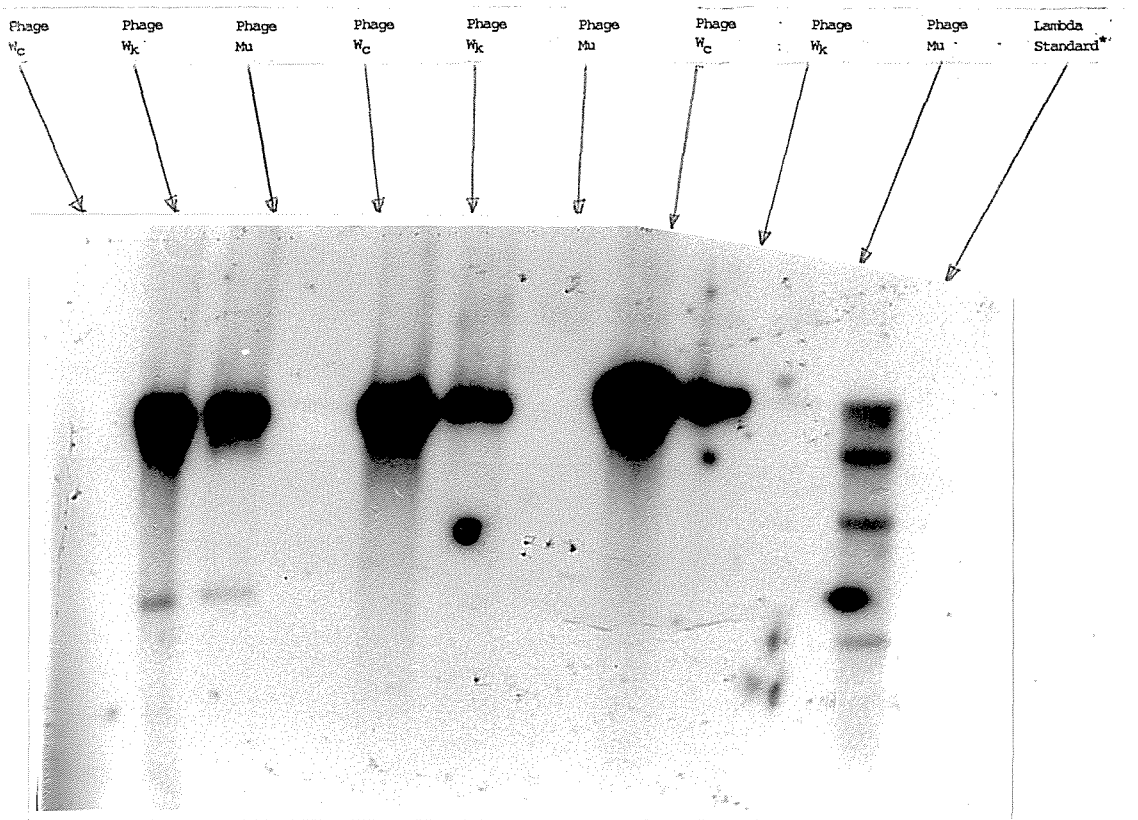
The following day, the bag was removed from the water and cut open. The dried gel was removed, still on the filter paper, and washed in 2 x SSC + 1 % Sarkosyl for 15 minutes, then washed for 3 x 15 minutes in 2 x SSC. The dried gel (without the filter paper) was then removed (very carefully!), dried on paper towels and wrapped in Glad-wrap.

An autoradiograph was prepared by lying the gel, still in its Glad-wrap, onto a piece of X-ray film and sandwiching this between 2 intensifying filters in an X-ray cassette. The cassette was stored at -70°C for 2 days (about 10^4 cpm or more) or as long as a week (for less than 10^3 cpm), before developing the film.

Probing Restriction Digests for DNA Homology.

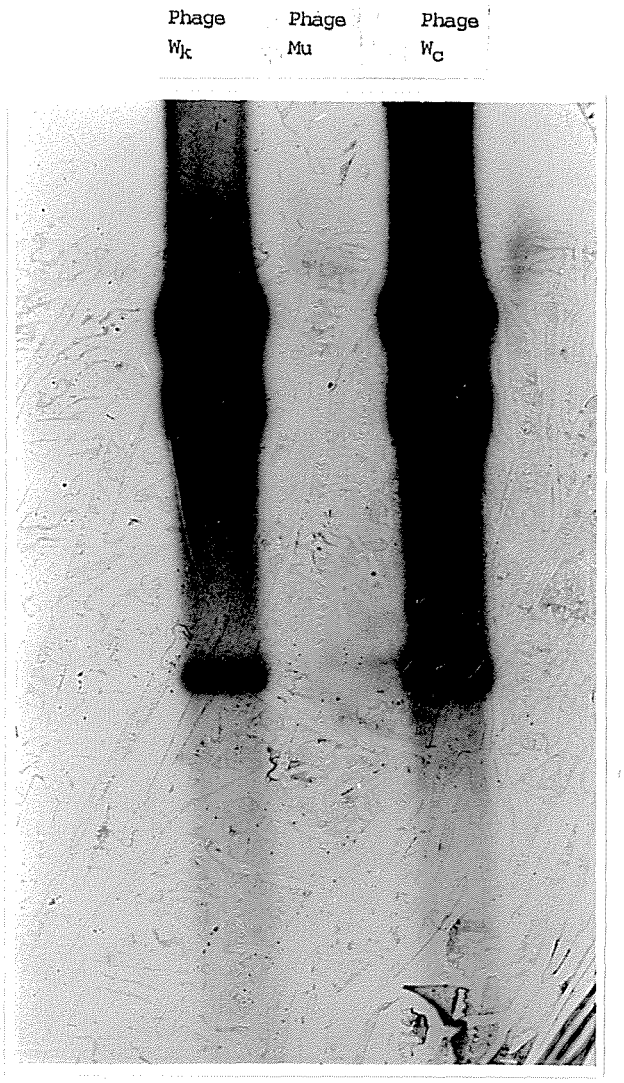
As the following autoradiographs demonstrate, there is almost total homology between the DNA from the W_o and W_k phages. At the relatively high level of stringency detailed above, no appreciable difference was detected. In view of the identical nature of the restriction digests done, it would not seem premature to propose that the 2 phages are 2 forms of the same phage.

Plate #5. On the right, 2 µl each of undigested Mu, W_k, and W_o DNA (reading from right to left). In the centre, 2 µl each of Mu, W_k, and W_o DNA (reading from right to left) digested for 2 hours at 37°C with EcoRI, and on the left, 2 µl of DNA in the same order digested for 2 hours with Sal I. This gel was run on a 1 % gel at 45 volts for 10 hours and visualised by soaking the gel in 0·5 % Ethidium bromide for 15 minutes before photographing under UV light with a MP.4 Polaroid Land Camera. (See Plate #4, but note that the film has been reversed - right in the former photo is now left - by converting the negative to a print.) The gel was then dried onto nitrocellulose filter paper and probed with 1 µl of phage W_o DNA labelled with radioactive CTP (a total of approximately $1{\cdot}5 \times 10^3$ cps). The dried gel was visualised by exposing it to an X-ray film for 3 days. The significant feature of this gel is that, while the small fragments from the W-phage digests have been detected, there is no apparent hybridisation to the Mu DNA, not even to the fragment in the EcoRI digest which contains the G-region. This implies that there is no significant homology between the W-phage and Mu DNA.



*Cut with Hind III

Plate #6. This autoradiograph shows 2 µl each of W_k, Mu and W_c DNA (reading left to right) digested with Hind III and EcoRI, then run at 45 volts in 1 % agarose for 10 hours. The gel was visualised by soaking the gel in 0·5 % Ethidium bromide for 15 minutes before examining under UV light. This gel was not photographed, but see Plate #1 for a comparison. The gel was then dried onto nitrocellulose filter paper and probed with 1 µl of phage W_c DNA labelled with radioactive CTP (a total of greater than 2·0 x 10^3 cps). The dried gel was visualised by exposing it to an X-ray film for 2 days. The significant feature of this gel is that even at the very high loading of label, no hybridisation of the probe to the Mu DNA can be seen. This gel was hybridised at a lower temperature than the preceding one (Plate #5) in an attempt to detect a low level of homology. It seems unlikely, therefore that the W-phage and phage Mu have any regions of significant homology.


Phage
W_K
Phage
Mu
Phage
W_C

Total DNA Isolation from Escherichia coli.

Sufficient genomic DNA for examination can be readily isolated from a small sample of cells. The E.coli strain was inoculated into 2 millilitres of BHI broth and incubated overnight at 37°C.

The following day, the culture was spun down in a type A Sorvall benchtop centrifuge, washed in 0·5 millilitres of 50/20 TE buffer, and spun down as before. The pelleted cells were drained and resuspended in 0·25 millilitres of 50/20 TE buffer.

These cells were lysed by adding 10 microlitres of lysozyme and incubating at 37°C for 30 minutes. The lysozyme should be fresh and can be made by adding 1·5 milligrams of lysozyme powder to 0·2 millilitres of 50/20 TE buffer.

After this initial incubation, 10 microlitres of proteinase K (made fresh with 2 milligrams of powder in 0·13 millilitres of 50/20 TE buffer), and 50 microlitres of Sarkosyl (made fresh with 45 millilitres of powder in 0·75 millilitres of 50/20 TE buffer) were added. This mixture was incubated overnight at 50°C, by which time lysis was complete, and the culture was totally clear.

The following day, the DNA was sheared with a sterile 20-gauge needle and 1 millilitre syringe. The DNA was extracted with an equal volume of equilibrated phenol and chloroform, as detailed in the description of the extraction of phage DNA. As a second step in this procedure a back-extraction was made of the first phenol extract with 50 microlitres of H_2O. This was combined with the aqueous phase from the first extraction.

The purified DNA was precipitated with 1/20th the total aqueous volume of 1 molar NaCl, plus twice the aqueous volume of absolute ethanol. Precipitation was allowed to take place over a period of 2 hours to

overnight at -20°C. The following day, the precipitated DNA was spun down as detailed previously, dried well, and dissolved in 50 microlitres of 10/1 TE buffer. The total genomic DNA was then ready for use.

Probing Genomic Digests for the Presence of Prophages.

The DNA prepared as described above was digested with various enzymes (listed below) and run on a gel in the same manner as the phage DNA and in paralell with phage DNA. The object of this was to probe the genomic digest with the labelled phage DNA to see if the number of copies of the prophages could be determined.

To this end genomic DNA was prepared from an E.coli K12 strain AT2535, which was used as a negative control. A strain AT2535 which had been used as the recipient in a mating with F-2-3-27 and which had been shown to have recieved a W_o prophage was used as one test strain (see Experimental section 2, cross #14). Since this mating had been interupted at an early stage, it was assumed that only a single prophage had been sent across. This appears to have been the case, since it proved possible to remove the prophage from this strain by selecting for a nearby marker in a conjugation cross (see Experimental section 2, cross #15). 2 strains of E.coli W were also prepared for testing, ATCC 9637 (E.coli W wild type), which carries both W_o and W_k prophages and F-2-3-27, which carries only the W_o prophage or prophages. This variety of lysogeny might enable some information to be gained on the number of prophages in the W-strains.

Preliminary work with these strains indicated that further investigation should enable a definitive statement to be made regarding the number of copies of the W_o and W_k prophages which are present in these strains of E.coli. Unfortunately time precluded this line of

inquiry being carried to a conclusion.

DISCUSSION AND CONCLUSIONS.

Escherichia coli strain W expresses a very tight restriction of most coliphages. That the inability of coliphages to propagate on this strain is due to restriction rather than some other mechanism has been demonstrated by observation of the degradation of labelled invading phage DNA [Pizer et al. 1968]. This ability to restrict could be shown to be due in part to a type I hsdS/hsdR/hsdM restriction and modification system (see Experimental section 1). This restriction system was isolated in the course of the work detailed in this thesis and characterised to a minor degree; it appears to be similar to the type I restriction / modification systems operating in other restricting strains of E.coli, such as E.coli K12 and E.coli B. Like the restriction systems in the E.coli strains just mentioned, the hsdS/hsdR/hsdM loci in E.coli strain W are located at approximately 0 minutes on the E.coli chromosomal map. The restricting and modifying activities of these loci were transferred in conjugation crosses and demonstrated in recipient strains (see Experimental sections 1 and 2).

It was also found that the hsdS/hsdR/hsdM alleles could be distinguished from another locus coding for restriction. This second restriction is associated with the transfer and incorporation of the W-phage in conjugation crosses using E.coli C strain recipients. This fact supported claims by earlier workers [Glover and Kerszman 1967, Pizer et al. 1968, Jamieson 1971] that the W-phage, which are lysogens of E.coli strain W, are associated with a restriction system. The W-phage associated restriction system is much more efficient than the type I restriction system present in E.coli W and other E.coli strains. This is

due to the fact that the phage associated (or phage-coded) restriction system does not have an associated modification system. Phage which do initiate a successful infection of cells carrying the hsdS/hsdR/hsdM system are modified and thereafter propagate freely on the particular strain for which the modification is specific. This type of behaviour has never been observed when the host cells are carrying the W-phage coded restriction system and restriction is therefore unusually complete.

The W-phage coded restriction system appears to require a host factor in order to be expressed. The nature of the proposed host factor is unknown but is implied by the inability of some E.coli strains to express restriction although they are lysogenic for the W_e or W_k phages. The non-expression of restriction is not likely to be due to a change in the phage, since progeny phage released from these non-restricting lysogens are capable of conferring restriction on hosts of a suitable strain. A second piece of evidence for the involvement of a host factor in the expression of the W-phage coded restriction was the observation that a nitrosoguanidine-induced mutant of E.coli W lost the ability to express the W-phage type restriction but still released phage which were viable and which could confer the typical W-phage associated restriction on cells which became lysogenic for these phage [Pizer et al 1968]. The continued ability of phage from the non-restricting E.coli W to confer restriction on new hosts implies that the gene or genes inactivated by the nitrosoguanidine treatment were part of the host genome rather than of the phage genome.

It was intended that the W-phage coded restriction system should be examined as the major thrust of this thesis. Examination of the role of the prophage(s) in restriction in E.coli W proved difficult because of

the presence of at least 2 different prophages, to wit, W_c and W_k. Two alternate methods of examining the prophages were either to separate them in E.coli W by elimination of one or the other prophage by recombination, or to separate them by sending the prophages singly into another (prophage-free) E.coli strain by conjugation using an E.coli W Hfr. The first method proved impractical but in the course of the second method (detailed in Experimental section 2), considerable evidence was obtained as to the location of the insertion sites of the W_c and W_k prophages.

It had been found that the W_k prophage had a strong linkage to the pro A,B loci and therefore the prophage insertion site must be close to 6 minutes on the standard E.coli K12 gene map. Further work (Conjugation crosses #7, 8, 9, 10, 11, 12 in Experimental section 2) revealed that the W_k prophage was transferred as an early marker by Hfr strains derived from the E.coli K12 strain Hfr808. The F insertion site in this Hfr strain is approximately 5¾ minutes implying that the insertion site of the W_k prophage is within the 4 to 5 minute region. This estimate was confirmed by means of a 3-factor cross (Experimental section 3), confirming a position of approximately 5 minutes.

The W_c prophage was more difficult to map. Unlike the W_k prophage, which was first tentatively located by its elimination from E.coli W, the W_c prophage was not observed to be eliminated by recombination. In conjugations involving an E.coli strain W Hfr and transfer of the W_c prophage to an E.coli K12 recipient, the W_c prophage was observed to show strong linkage to the pyr B locus which maps approximately at 96 minutes. (Conjugation crosses #14 and 15 in Experimental section 2). It is possible that there may be more than 1 copy of the W_c prophage in E.coli

W. This would explain a number of anomalous results regarding apparent linkage relationships of the W_e prophage with other selected markers.

It has been suggested that the W_e and W_k phages are in fact the products of a single phage producing different phage phenotypes by genetic switching. The behaviour of the W_k phage when plated on different hosts is certainly characteristic of genetic switching (see Figure 2). However, there is no evidence so far observed to suggest that genetic switching occurs with any great frequency during normal lysogeny; cells cured of W_k have not been observed to release phage of the W_K type (see Conjugation crosses #3 and #5 in Experimental section 2). In agreement with the assumption that the 2 W-phages are identical are 3 observations. First, that W_k can undergo conversion to a W_{ke} type which is indistinguishable from a W_e. Second, that either W_k or W_e individually are capable of causing a host cell to express the W-type restriction (see crosses II and III, Experimental section 2) implying that those genes as well as the genes controlling phage structure are common to both phages. Third, and most telling, is the fact that the 2 phages are indistinguishable at the genomic level, even when cut in restriction endonuclease digests with multiple enzymes.

Given that the only basis for distinguishing the W_e and W_k phages is their specificity for different hosts and considering the similarity of the W-phages to phage Mu (which has a gene-splicing mechanism controlling host-specificity and yielding host-range alternation similar to that observed in phage W_k) it was considered worthwhile to test for any homology at the genetic level. This was tested as described in Experimental section 4.

Total genomic DNA from the Mu phage failed to migrate through the agarose gels at the same speed as the genomic DNA from the 2 W-phages. This indicates a difference in molecular weight. It was found that both W-phage DNA samples had a genomic weight eqivalent to approximately 45 kilobases (kb), as opposed to a weight for the Mu DNA sample of approximately 38 kb, a figure which is in agreement with published values [Abelson et al. 1973, Bukhari et al. 1977]. In addition to this observation it was found that while the fragments of Mu DNA obtained with restriction endonuclease analysis were in agreement with published maps [Bukhari et al. 1977] the patterns obtained with the W-phage were completely different from phage Mu (see plates 1 - 4). As previously mentioned these restriction endonuclease digests did not reveal any differences between phage W_c and W_k.

The restriction endonuclease digests were complemented by probing the gels with labelled W_c DNA. This revealed no significant homology even at very high loadings of radioactivity and lowered specificity. As was the case with the restriction digests, however, no difference was observed between W_c and W_k. If genetic switching does occur in the W-phage, it must be by a mechanism that differs from that which elicits switching of the G or C-region of phages Mu and P_1, respectively. The similarity of W_c and W_k does raise an interesting point, however. The fact that both W-phage are indistinguishable at the genomic level implies that the ammount of genetic material that is different must be very small, yet W_k has the genetic information for 2 forms of attachment apparatus, one form of which allows it to duplicate the W_c host attachment pattern. The easiest way to reconcile these facts is to postulate some form of genetic switching, presumably active at low level frequency, sufficiently low,

such that, when W_c phage are plated on E.coli K12, the alternate host range mutant type ($W_{c'}$) predominates among the observed plaques.

So, to summarise, E.coli strain W expresses 3 restriction systems. These are the hsdW type I restriction/ modification, a modificationless restriction of phages P_1 and Lambda associated with the W-phage, and a system restricting the T-phages (see Experimental section 3), about which little is known.

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