The construction and use of \( \lambda \) vectors in molecular cloning.

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Voor mijn ouders
De techniek staat voor niets,
de wetenschap voor een raadsel.
Foreword

This thesis is based on work conducted by me in the department of Biochemistry, University of Leicester, during the period between August 1978 & August 1981 under supervision of Professor dr. W.J.Brammer.

The idea to introduce a chi-site into AM167 originated in the department of Molecular Biology of the University of Edinburgh, where my supervisor worked for many years. All experiments and ideas to construct AM141 and other strains in this thesis were designed and carried out by me unless otherwise stated in the text or by references.

None of this work has been submitted for another degree in this or any other University.
Acknowledgements

Everyone reading this thesis will become aware of how much I have enjoyed doing this work.

This is in the first place thanks to my supervisor, Bill Brammar, who has created such a pleasant atmosphere in the lab, encouraging discussion and channeling so many far-fetched ideas into fruitful experiments.

Many thanks also to the people in the lab, to Judy, Jack, Mark, Ross, Chris, Barry, Nigel, Chris, Alistair, Liu & Jean and to people in Genetics and the ICI Joint lab for strains, helpful discussions and/or cheering-up sessions on my off-days.

A special word for my friends Dave, for sharing so many strains and ideas (crazy ideas, my foot) and Anne, without whom life in the lab would not have been the same.

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Abstract

A multipurpose λ vector was constructed which can be used for cloning DNA fragments of about 20 kb generated by restriction enzymes EcoRI, HindIII, BamHI and enzymes producing the same sticky ends (e.g. BglII, BclI, NheI and Sau3A). Recombinants can be selected by their Epi− phenotype and their propagation is facilitated by the presence of a chi-site. This choice of enzymes simplifies the cloning procedure considerably and makes this vector particularly suitable for the establishment of gene banks. It can be used in simple cloning experiments, selecting recombinants on a λ2lysogen as well as for highly efficient cloning after removal of the vector's central fragment and size selection of the donor DNA.

This vector can also be used as an expression vector when cloning into the vector's BamHI sites using the powerful λ promoter for leftward transcription, nL, and the phage's antiterminator function, M.

Recombination-deficient hosts for the propagation of recombinants have been investigated to minimize undesired genetic rearrangements.

The absence of restriction targets for several enzymes in the vector arms led to attempts to clone the genes for these enzymes and their modification enzymes. When these failed, the genes of Providencia stuartii 164 were used as a model system to study the behaviour of such genes when carried on a λ vector. These genes turn out to be highly unstable in such a situation and experiments suggest a possible negative influence of the modification activity on host and viral functions.
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Notes

1) Throughout this thesis I have used recA, recBC, chi etc.;
   s.s. this should be recA\textsuperscript{−}, recBC\textsuperscript{−}, chi\textsuperscript{−} etc. as opposed to recA\textsuperscript{+}, recBC\textsuperscript{+},
   chi\textsuperscript{+} etc.

2) sup\textsuperscript{F} = su III; sup\textsuperscript{E} = su II.

3) amp (in text) or Ap (in diagrams) = ampicillin
   tet " or Tc " = tetracyclin

4) In drawings and diagrams the notations of restriction targets are as
   described in M-27; those of substitutions as stated in fig.1-3 (p.1-11).

5) m.o.i. = multiplicity of infection
   e.c.p. = efficiency of plating

6) ON : Overnight culture

7) rT : roomtemperature

8) LB : Luria broth
   L + amp : LB + ampicillin (20 ug/ml)
   L-plates: LB + 1 % agar

9) D : Dalton
Introduction

Cloning vectors

The last decade has seen the rapid development of cloning techniques to study gene structure, the control of gene expression and gene products of both pro- and eukaryotes. Essential to these studies has been the development of a whole range of suitable vector molecules, i.e. independent replicons which can carry and propagate DNA from other organisms. The choice of vector depends on the reason for cloning the genes of interest. As vectors, either viruses e.g. E.coli virus λ (a 'bacteriophage' or 'phage', Hershey (ed), 1971) or plasmids (e.g. pBR322, Bolivar et al, 1977) have been used.

Vectors such as pBR322, or its derivative pAT153, have a high copy number and can be amplified using chloramphenicol, allowing high yields of the cloned DNA for subsequent study. These plasmid vectors are ideal for the propagation of small DNA fragments. Large plasmids however tend to be unstable and efficient recovery of the DNA can be difficult.

Expression vectors have been developed in which the DNA is cloned downstream of a powerful promoter, which preferably can be regulated. Examples of these are the E.coli lac and trp promoters and the phage λ P_L promoter. The P_lac and P_trp promoters are controlled by metabolite levels, while P_L is under control of the λ repressor, the product of the phage oI gene. These promoters have been used both in λ and plasmid vectors using in vivo and in vitro cloning techniques, with suitable targets for restriction enzymes downstream (Backman et al, 1976; Roberts et al, 1979; Burt, 1981).

The level of expression from these promoters or from the gene's own promoter can be monitored by fusing the E.coli lacZ gene to the operon in question. Expression of the lacZ gene can be visualised on Xg or lac-McConkey indicator plates (M-12), the exact level of activity being readily determined with a spectrophotometric assay for β-galactosidase, the product of the lacZ gene.
(Mitchell et al., 1976; Casadaban & Cohen, 1980; and see Burt, 1981, for an extensive discussion).

**Recognition of recombinants**

Recognition of recombinants following a cloning experiment originally involved laborious screening. If the target used for cloning lies in a known gene, one can screen for insertional inactivation of that gene, e.g., for loss of \( \lambda_{\text{red}} \) function (such \( \lambda_{\text{red}} ^{-} \) phages fail to grow on an E.coli \( \text{polA} \) mutant strain) or loss of drug resistance (e.g., tetracycline resistance (\( \text{tet}^{P} \)) of pBR322).

Positive selections have been developed recently. Roberts et al. (1980) fused the \( \text{tet} \)-gene to a \( \lambda \) promoter, \( \text{P}_{\lambda} \), adjacent to the \( \lambda_{\text{CI}} \) gene. The \( \lambda \)-repressor also acts on \( \text{P}_{\lambda} \). Insertional inactivation of the \( \lambda_{\text{CI}} \) gene will lead to expression of \( \text{tet} \) from \( \text{P}_{\lambda} \); recombinants are \( \text{tet}^{P} \), while colonies containing the original vector will be \( \text{tet}^{S} \).

**Gene banks**

To isolate the gene(s) of interest, we can use several methods.

We fragment the DNA of the appropriate organism using (a) restriction enzyme(s) and mix these fragments for ligation with vector DNA, cut with the same enzyme(s) or (an) enzyme(s) producing the same sticky ends (‘shotgun’ experiment).

Alternatively we can produce fragments with blunt ends by shearing the DNA, using an enzyme producing flush ends or by filling in the sticky ends using DNA polymerase. Synthetic linkers can be fused to these blunt ends using \( T_{4} \)-coded DNA ligase, carrying the sequence for an enzyme (usually EcoRI) or long tails of a particular nucleotide (e.g., poly-dA) can be added using terminal transferase (Clarke & Carbon, 1975; Maniatis et al., 1978). In the former case we have to protect the DNA to be cloned with (EcoRI) methylase prior to ligation of the linkers, so we can open up the (EcoRI) restriction site in the linker before ligation onto the vector DNA, cut with the same
enzyme (EcoRI).

The fragments used for the addition of synthetic linkers, have usually been size-selected on a sucrose gradient to optimize the number of recombinants and avoid ligation of more than one hitherto unlinked donor fragment onto one vector molecule.

The reasons for the procedure using EcoRI linkers is in the case of $\lambda$ partly a historical one. Most of the original $\lambda$ vectors are designed for use with EcoRI, due to the existence of an E.coli strain carrying a plasmid producing this enzyme (Yoshimori, 1971). Using a particular enzyme one runs the risk that the gene(s) of interest carry targets for that enzyme or is (are) carried on too long a fragment to be cloned (see further). Using a different fragmentation method as described above and providing the fragments with EcoRI linkers allows you to use a different enzyme in conjunction with an EcoRI vector. The vector DNA is prepared with complementary sequences (e.g. EcoRI sticky ends or poly-dT tails) and mixed with the donor DNA for ligation.

The reason for the use of synthetic linkers for plasmids is to avoid re-annealing of the vector's sticky ends to each other rather than to the donor DNA. (The Jacobson-Stockmayer equation ($j = (h/L)^{3/2}$) predicts that the initial rate of reaction leading to monomolecular cyclization or bimolecular joining is dependent on the size of the DNA. The smaller the vector, the higher the rate of the former at similar DNA concentrations, leading to loss of vector DNA molecules as potential carriers of the donor fragments).

Since the discovery of the presence in eukaryotic DNA of intervening sequences, which cannot be processed in E.coli, much cloning has involved the use of cDNAs, made by reverse transcription of the mRNA of the eukaryotic cell. The double-stranded cDNA is then provided with synthetic linkers as described for the preparation of genomic DNA for cloning.

The formula $N = \ln(1-x)/\ln(1-f)$, which is discussed in M-28 for E.coli, allows you to calculate the number of recombinant clones needed to cover the
whole genome of a certain organism. It is dependent on the average size of the cloned fragment and the total length of the genome to be covered \( f; x \) is the probability of finding a given gene. Using this formula we can calculate that we need about \( 10^6 \) clones with inserts of 10-15 kb to cover the genome of a mammal with a 99\% probability. The use of large fragments for cloning decreases the chance of cutting in the desired gene as well as the number of recombinants needed.

The pool of recombinant molecules covering the entire genome of a certain organism is called a gene library or gene bank. To establish a gene bank of a large genome we need a vector which can carry and easily propagate large DNA fragments. This includes an efficient recovery of the DNA after ligation. These considerations leave us with two vector alternatives: \( \lambda \) or a special type of plasmid vector, the cosmid (see further).

A gene bank allows you to find a given gene, but also allows you to find partially overlapping clones, which will give you information about the DNA adjacent to the gene of interest. Other DNA fragments with sequences homologous to the clone of interest can thus be isolated (see further discussion).

**Cosmids**

The in vitro packaging technique which has been developed for \( \lambda \) (Sternberg et al, 1977; Hohn & Murray, 1977) has led to the construction of cosmid vectors. Cosmids are plasmids carrying the cohesive ends of \( \lambda \) (cos-site), DNA sequences involved in recognition by the \( \lambda \) packaging functions. Extracts from 2 heat-inducible \( \lambda \) lysogens, whose DNA is trapped in the E.coli chromosome, contain all the functions necessary to package DNA of 40-50 kb carrying the \( \lambda \) cohesive ends, independent of the origin of the DNA in between those sequences.

Since the cosmid vector itself can be small, cosmid vectors can carry large inserts (up to 48 kb) (Meyerowitz et al, 1990). Plasmids themselves are not really suitable for gene banks. DNA isolation of large plasmids as well as transformation with such DNA is difficult and large plasmids
are often highly unstable \textit{in vivo}. With cosmids one avoids the first problem using the \textit{in vitro} packaging technique. DNA recovery using this technique is at least a 100-fold more efficient than transformation with CaCl$_2$-treated cells (Yandell & Fira, 1970). As far as stability of these recombinant cosmids is concerned, reports have been variable and it seems to depend on the user and possibly the origin of the cloned DNA.

\textbf{Bacteriophage \lambda}

The DNA of bacteriophage \lambda is a very large molecule (ca. 50 kb) compared to that of plasmid vectors like pBR322 (4.4 kb). The latter often have only one target for a particular enzyme (based on a recognition sequence of 6 bp (as is the case with many of the restriction enzymes) there will be a target for such an enzyme once every 4$^6$ bp (i.e. every 4-5 kb on average). Two approaches have been used to remove unwanted restriction targets from the \lambda genome.

The first one is removal of targets by enrichment for phages carrying point mutations which change those recognition sites. The availability of an \textit{E. coli} strain carrying the EcoRI restriction and modification system, allowed the \textit{in vivo} enrichment for mutant \lambda phages, which had lost targets for this enzyme (Murray & Murray, 1974, 1975; Rambach & Tiollais, 1974; Thomas et al, 1974). This is the reason why a lot of the earlier \lambda vectors have been developed for use with EcoRI. Using \textit{in vitro} enrichment techniques, sites for other restriction targets have since been removed (e.g. for BamHI: Klein & Murray, 1979; Rim et al, 1990). The latter involves repeated partial digestion of the phage DNA followed by \textit{in vitro} packaging, amplification of the phage progeny, DNA preparations, partial digestion etc. This technique uses a lot of enzyme and is labour intensive, but is sometimes unavoidable.

The second method for the removal of undesired restriction targets is deletion of the DNA in that area or substitution with other DNA. Where possible people have used deletions or turned to related lambdoid phages, such as \lambda 34, 21 and \phi 80. The existence of these phages with a similar organisation of the genome
as \( \lambda \), has allowed the replacement of vital areas on the \( \lambda \) genome by those of these related phages with analogous functions, thereby removing (or introducing) (a) certain target(s).

The \( \lambda \) genome

We can describe the \( \lambda \) genome basically as a DNA molecule containing parts which are essential for lytic growth (unshaded regions, fig.I-1) and areas which are not (shaded regions, fig.I-1). This is depicted in fig.I-1:

The location of two of the most used substitutions, the immunity regions of \( U_{24} \) and 21, is also shown in this figure.

The shaded regions contain genes involved in recombination and the lysogenic state of the phage.

The genes \( \sigma \) and \( \Pi \) are involved in phage replications, genes \( \sigma \) and \( \Pi \) in cell lysis, while \( \Lambda-J \) code for the structural genes of the phage. \( \sigma \) is a late transcription control function (see below).

Transcription of \( \lambda \) phage genes during lytic growth

\( \lambda \)'s early transcription starts from two promoters in the immunity region, \( \Pi_{L} \) for leftward transcription and \( \Pi_{R} \), reading rightwards: see fig.I-2 (page I-7).

From \( \Pi_{R} \) the \( \Pi \)-gene is transcribed. The \( \Pi \)-gene product interacts with the E.coli RNA polymerase thereby allowing it to read through the stop signals \( \Pi_{L} \), \( \Pi_{L1} \) and \( \Pi_{L2} \).
Fig. I-2: Transcription of λ-phage genes during lytic growth

I. Transcription immediately on induction or infection. In the absence of λ-phage's N protein, leftward transcription terminates efficiently at site t4, while rightward transcription terminates inefficiently at site t5, beyond the cro gene. About 20% of the rightward transcripts escape termination at site t6, and proceed through genes O and P to be stopped at site t7 (thin arrow). II, after expression of the N gene, transcription is influenced to ignore sites t4, t5, and t6. Leftward transcription leads to expression of the recombination (red) and integration (int) functions; rightward transcription proceeds through the O, P, and Q genes, leading to DNA replication and the activation of late transcription. III, The product of the Q gene activates late transcription from promoter Pα to proceed through genes S and R, governing cell lysis, and through genes A to J, since the genome is circular at this time.

The remainder of the N-operon contains genes involved in the lysogenic state and the phages recombination genes red and gam. The former can be deleted without consequences for the lytic phase. The recombination genes red and gam can be deleted under certain conditions as explained later. The N-gene can be removed from the phage if we also remove the strong terminator tP2 (by a nin-deletion (shaded region between the P and Q genes, fig. I-1)), allowing late transcription, stimulated by the Q-gene product, to proceed.

Late transcription produces the lysis functions (S and R) and the structural proteins of the mature phage particle (A-J). The OP region contains the origin of DNA replication and genes involved in this replication.

The replaceable region

From the above we can conclude, that we can replace the DNA between J and pR, called the replaceable, or non-essential region, and still produce potentially viable phage. However the λ packaging system poses both a minimum and a maximum size limit on the DNA (about 78-109% λ+ length (Bellett et al, 1971; Weil et al, 1972). This means that the non-essential phage DNA cannot simply be
deleted, but has to be replaced by some other DNA (both ϕ80 and E.coli DNA substitutions have been used extensively (for a survey see Williams & Blattner, 1980). The packaging system also puts a size limit to the DNA to be cloned. Concluding, we can either replace this central region with other DNA or insert foreign DNA anywhere in this region without endangering the phage's lytic life cycle.

λ vectors

Insertion vectors (one target) and replacement vectors (two or more targets) have been developed for use with EcoRI, HindIII and recently, since the start of this work, BamHI (Klein & Murray, 1979). Insertion vectors usually carry large deletions or substitutions, removing up to 20% of the phage's DNA, thereby allowing inserts up to about 13 kb. The target is preferably in a gene, whose function we can recognise (insertional inactivation, e.g. in the ci gene (changing the phenotype from turbid to clear (λNM607; see table I-I) or in red (λred- phage fails to grow on polA hosts (e.g. λCh12)). Where no such recognition is available pel- hosts can be used in conjunction with small vectors. Only phages of nearly 100% λ length can grow on such a strain (Scandella & Arber, 1974, 1976; Emmons et al, 1975).

Replacement vectors can be either large or small. Depending on the position of the targets, smaller or larger fragments can be cloned, with or without an easy selection for recombinants (e.g. λgt (Thomas et al, 1974) and its derivative λgtWES (Enquist et al, 1976), λCh4 and its derivative λCh4A (Blattner et al, 1977; Williams & Blattner, 1979) and λNM781 (Borck et al, 1976); see table I-I).

Recombinants of λgt(WES) will have to be screened for loss of integration function while λCh4(A) uses the already discussed easily recognisable E.coli lacZ gene on the replaceable region for identification of recombinants. λNM781 carries the supE gene of E.coli on the central fragment and can be used in conjunction with a lac am host for recognition of recombinants. Other genetic
markers such as \textit{trp}, \textit{bio}, \textit{ara} & \textit{surF} have been used.

When a high number of recombinants is required, the central fragment in these replacement vectors is removed from the phage DNA prior to ligation, using a sucrose gradient to separate the arms from this fragment. The vector arms are annealed prior to this separation step.

Table I-I gives a brief survey.

Table I-Ia shows some of the substitutions and deletions used in \textit{\lambda} vectors.(p.I-10)

Table I-Ib lists some vectors and the tests used to recognise the recombinants of these vectors.(p.I-12)

A simplified map of these phages is shown in fig I-3.(p.I-11)

\textit{\lambda}M' vectors have been developed by H. Murray (Edinburgh, GB)

\textit{\lambda}Ch vectors have been constructed by F. Blattner (Madison, USA)

\textit{\lambda}rt: Thomas et al, 1974

\textit{\lambda}B': Rambach & Tiollais, 1974
Table I-Ia  Frequently used substitutions and deletions in \(\lambda\) vectors

<table>
<thead>
<tr>
<th>Region</th>
<th>Deletion</th>
<th>Substitution</th>
<th>Example(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>left arm</td>
<td>(('b')^\uparrow) (1)</td>
<td>none</td>
<td>(\lambda) Ch12</td>
</tr>
<tr>
<td>central area</td>
<td>((\alpha)^\uparrow)(KH54)</td>
<td>(\delta80)</td>
<td>(\lambda) Ch4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\lambda) lacZ</td>
<td>(\lambda) Ch12 (\lambda)'B'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\lambda) supE</td>
<td>(\lambda) NM781</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\lambda) bio</td>
<td>(\lambda) Ch4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\lambda) imm21</td>
<td>(\lambda) NM540</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\lambda) imm434</td>
<td>(\lambda) NM607</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(\lambda) imm80</td>
<td>(\lambda) Ch2</td>
</tr>
<tr>
<td>right arm</td>
<td>((\text{nin})^\uparrow)</td>
<td>(\lambda) NM567 (\lambda) NM781 (\lambda) st</td>
<td>(\lambda) Ch4</td>
</tr>
<tr>
<td></td>
<td>((\text{GSP})^\uparrow)(nin5) (\lambda) bio236 (KE54) (\lambda) (nin5) (\lambda) (OSP)</td>
<td>(\lambda) NM607 (\lambda) NM781</td>
<td></td>
</tr>
</tbody>
</table>

1) \(('b')^\uparrow\): in vivo deletions: e.g. \(b2\), \(b538\)  
or in vitro deletions e.g. \((\text{sri} \lambda 1-2)^\uparrow\), \((\text{sbi} \lambda 2-3)^\uparrow\)

2) accompanied by a duplication in the left arm

3) phenotype of the phages shown as examples:

\(\lambda\) NM540 \(\lambda (\text{sri} \lambda 1-2)^\uparrow\text{imm21}(\text{nin}5)^\uparrow\text{shn} \lambda 6^0\)
\(\lambda\) NM567 \(\lambda (\text{srI} \lambda 1-2)^\uparrow\text{srI} \lambda 5^0\text{imm434}\) \(\text{srI} \lambda 4^0(\text{nin}5)^\uparrow\text{srI} \lambda 5^0\)
\(\lambda\) NM607 \(\lambda\) b538 \(\text{imm434}\) \(\text{srI} \lambda 5^0\)
\(\lambda\) NM781 \(\lambda\) supE \(\text{cl357}\) \(\text{srI} \lambda 6^0(\text{nin}5)^\uparrow\text{srI} \lambda 5^0\)
\(\lambda\) NM780BV2 \(\lambda\) sbhi\(\text{srI} \lambda 2-3)^\uparrow\text{red3} \text{imm21}(\text{nin}5)^\uparrow\text{shn} \lambda 6^0\)
\(\lambda\) Ch2 \(\lambda\) plac5(\text{att}80-\text{imm}80(\text{KH}53)^\uparrow)(\text{nin}5)^\uparrow\text{OSP80})
\(\lambda\) Ch4 \(\lambda\) plac5 \(\text{bio}256(\text{KH}54)^\uparrow(\text{nin}5)^\uparrow\text{OSP80})
\(\lambda\) Ch12 \(\lambda\) (dup1) \(\text{b}109(\text{KH}54)^\uparrow(\text{nin}5)^\uparrow\text{OSP80})
\(\lambda\) Ch20 \(\lambda\) b1007 \(\text{imm}434\) \(\text{srI} \lambda 4^0(\text{nin}5)^\uparrow\text{shn} \lambda 6^0\) \(\text{srI} \lambda 5^0\)
\(\lambda\) st \(\text{srI} \lambda 5^0(\text{nin}5)^\uparrow\text{srI} \lambda 5^0\)
\(\lambda\)'B' \(\lambda\) plac5 \(\text{cl357}\) \(\text{srI} \lambda 6^0\) \(\text{srI} \lambda 5^0\)
Fig. I-3: A simplified map of some vectors, as listed in Table I-I. See text and Table I for details.
### Table I-1b: Examples of some vectors

<table>
<thead>
<tr>
<th>phage</th>
<th>relevant characteristics</th>
<th>enzyme</th>
<th>selection/screening for recombinants</th>
<th>cloning capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>λNM540</td>
<td>none</td>
<td>H</td>
<td>none</td>
<td>0-12 kb</td>
</tr>
<tr>
<td>λNM567</td>
<td>Spi⁺</td>
<td>E</td>
<td>Spi⁻ (1)</td>
<td>5-22 kb</td>
</tr>
<tr>
<td>λNM570E2V2</td>
<td>int⁺</td>
<td>E</td>
<td>int⁻ (2)</td>
<td>0-12 kb</td>
</tr>
<tr>
<td>λNM607</td>
<td>turbid</td>
<td>R</td>
<td>clear</td>
<td>0-11 kb</td>
</tr>
<tr>
<td>λNM781</td>
<td>supE</td>
<td>R</td>
<td>compl. lac ar (3)</td>
<td>2-15 kb</td>
</tr>
<tr>
<td>λCh2</td>
<td>lac⁺</td>
<td>R/ R+S</td>
<td>lac⁻ (4)</td>
<td>0-5 kb / 8-21 kb</td>
</tr>
<tr>
<td>λCh4</td>
<td>lac⁺ (bio⁺)</td>
<td>R</td>
<td>lac⁻ (bio⁻)</td>
<td>7-20 kb</td>
</tr>
<tr>
<td>λCh12</td>
<td>red⁺</td>
<td>R</td>
<td>Feb⁻ (5)</td>
<td>0-7 kb</td>
</tr>
<tr>
<td>λCh20</td>
<td>Spi⁺</td>
<td>R, H</td>
<td>Spi⁻</td>
<td>5-21 kb / 7-19 kb</td>
</tr>
<tr>
<td>λB⁺</td>
<td>lac⁺</td>
<td>R</td>
<td>lac⁻</td>
<td>1-14 kb</td>
</tr>
<tr>
<td>λgt c)</td>
<td>int⁺</td>
<td>R</td>
<td>int⁻ / int⁻ xis⁻ (6)</td>
<td>2-15 kb</td>
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</table>

a) R = EcoRI; H = HindIII; B = BamHI; most of these vectors can be used with XhoI and SalI as insertion vectors.

b) computer data from Williams & Blattner, 1980.

c) λgt.C is deleted for (srIL1-2)\(^\vee\); λgt.E is deleted for (srIL2-3)\(^\vee\).

---

1. selection on a (P2)lysogen, see M-20
4. plaques are white on Xg indicator plates: M-12
5. Feb⁻: failure to plate on polA or lig ts host strains: M-20
The Spi⁻ selection

A potentially useful selection is that for Spi⁻ recombinants (λNM567 or λCh20). Phages lacking the red and ram genes of λ grow on a host strain lysogenic for phage P2. Red⁺ram⁺ phages fail on such a host due to interference of the P2-old product, which interacts with red and ram (Sironi, 1969; Sironi et al., 1971; Zissler et al., 1971b). λNM567 and λCh20 however did not fulfil their promise as vectors. The problem with such Spi⁻ recombinants is that due to the absence of red and ram functions the phages grow very poorly. The red and ram genes are involved in recombination, which is a vital step in λ's life cycle. The packaging system will only package DNA which is flanked by two intact cos-sites. So DNA can be packaged from circular dimers, trimers etc., as well as from linear concatemers, but not from monomeric circular or linear species.

DNA replication and recombination in λ

Normal λ replication starts with θ type structures, switching to the rolling circle mode at a later stage (Skalka, 1974). The switch mechanism involves the λram gene. Both red and ram play a role in the rate of replication (Enquist & Skalka, 1973; Skalka, 1974). The switch from θ to rolling circle replication is inhibited by the E.coli recBC product, whose activity is counteracted by the λram gene product. In the absence of ram and the presence of recBC, no rolling circles can be formed and all packaging proceeds from circular dimers, formed via the phage's red system (which requires DNA synthesis, involving the host's polA and lig functions in addition to either DNA replication or the presence of the E.coli recA product) or the host's recAR pathway. A sbcA mutation, opening up the E.coli recE pathway, can replace the λred function and is believed to result in derepression of a DNA nuclease, exoVIII. Clark (1974) gives a model explaining the roles of the recBC and recE pathways and a third pathway, recF, in E.coli recombination. Gillen (1974), Stahl & Stahl (1977) and Stahl et al...
(1978) propose models for λ recombination, using host and phage functions. These possible pathways are summarised in fig I-4.

Fig I-4 Possible ways for λ recombination

(1) λ's normal recombination takes place via this pathway, involving λ red and gam functions. RecBC inhibits initiation of rolling circles, which activity is counteracted by λ gam function.

(2) E. coli's major recombination pathway is the recA recBC pathway; this pathway can be used by λ, but only efficiently when a chi-site is present.

(3) In the absence of λ gam λ follows a pathway involving E. coli polA and lig functions in addition to its own red function.

(4) In the absence of recBC E. coli can use the recF pathway by inactivation of a constitutive DNA nuclease, exoI (sbcB^-). This pathway is recA dependent and can be used by λ.

(5) An alternative pathway to recF is recE. This pathway is believed to work in the absence of recBC after derepression of a DNA nuclease, exoVIII.
The recE pathway has been reported to work in the absence of recA function. If the proposed action of sbcA (a function analogous to the red) is true, this would lead to the conclusion that the recE pathway, in the absence of recA, can act on replication intermediates like red.

The consequences of these complicated interactions are that λ red phages fail on host strains, mutant in DNA polymerase I (polA) or ligase (lig) functions, while λ red gam phages fail on polA, lig ts as well as recA hosts.

The chi-sequence, a crossover hotspot instigator.

The λ DNA is normally not a very good substrate for the E.coli recAB system, so λ red gam phages give very small plaques and low phage yields. But λ red gam phages spontaneously produce large plaque mutants (Henderson & Weil, 1975; Stahl et al, 1975). These mutants turn out to have acquired a mutation causing a chi-site (chi = crossover hotspot instigator). These hotspots can arise at four different loci in the λ genome (Stahl et al, 1975; 1980). Sprague et al (1978) showed this to have arisen in the case of a chiC mutation (in the λ cII gene) via a single basepair change.

These chi-sites occur naturally and at high frequency in the E.coli chromosome (Malone et al, 1978). Sequencing data on chiC and other chi-sites (Smith et al, 1980; Schultz et al, 1981) have shown a common sequence 5' GCTGCTGG 3' (or its complement or both) to be required for this hotspot activity. There is increasing evidence for Stahl's hypothesis that these sites are involved in recognition by the E.coli recBC product. Such sites therefore make the λ DNA, which is normally devoid of such sequences, a good substrate for this (major) recombination pathway in E.coli in the absence of red and gam.

Chi sites have no detectable activity when λ uses its own red pathway or the E.coli recE pathways.
Aim of this work

The initial aim of my work in Leicester, was to introduce such a recombinogenic chi-site into the EcoRI replacement vector λ NM567 (table I-a). Recombinant phages, which would be Spi⁻ and therefore easily selected on a (φ2) lysogen, would be able to use this chi-site for growth on a Rec⁺ host, giving a good phage yield. The choice fell on chiA, which is located in the left arm of the phage.

In addition, it was decided to introduce the shn 16⁰ mutation into the right arm, which would make this vector suitable for use with HindIII. Such a phage could be used as a vector for the establishment of gene banks in conjunction with two different restriction enzymes, EcoRI and HindIII, while the cloning capacity is large (15-20 kb fragments can be easily cloned).

The availability of λNM570BV2, the first BamHI insertion vector (Klein & Murray, 1979), allowed us to prepare λ WL57, the first λ replacement vector to be published for use with this enzyme (chapter 1, 2 and 3).

Problems with duplications in the left arm of one of the vectors prompted the search for recombination-deficient hosts to minimize the risk of such undesired genetic exchanges, without severely reducing the phage yield (chapter 4 and 5).

The absence of targets for HindIII, BamHI and SalI in the arms of the multipurpose vector λ WL57, lead us to investigate the possibility of cloning the genes for these restriction enzymes into λ. When these attempts failed, the restriction and modification genes of Providencia stuartii 164, known to be carried on a 4 kb HindIII fragment, were used as a model system to study their behaviour, when carried on a λ vector (chapter 6).
## Materials & Methods

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<td>- Preparation of bacterial DNA</td>
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</table>
V Preparation of phages, tests and DNA preparation

- Phage cross
- Sam enrichment
- Preparation of $\lambda$WL58 by marker rescue
- Preparation of $\lambda$WL37
- Complementation test
- lacZ test
- groP test
- groE test
- groN test
- Supernus test
- Test for the presence of the $\lambda$red and gam genes
- Test for the number of EcoRI targets in $\lambda$

- Phage preparations
  a) plate lysates
  b) 'blattner'
  c) liquid lysate

- Preparation of phage DNA
  1) concentration of the phage
     a) Pelleting of the phage by centrifugation
     b) PEG-precipitation
  2) purification of the concentrated phage
     a) CsCl step gradient
     b) CsCl equilibrium gradient
  3) preparation of the DNA
     a) pronase treatment
     b) phenol extraction
     c) DNA content
VI  In vitro manipulation
  - Restriction
    a) restriction reaction
    b) ethanol precipitation
    c) isopropanol treatment
    d) methoxyethanol extraction
  - Ligation
    a) ligation reaction
  - Phage recovery after ligation
    1) transfection with \( \lambda \) DNA
      a) competent cells
      b) transformation
    2) In vitro packaging of \( \lambda \) DNA
      a) preparation of in vitro packaging extracts
        (i) induction of the culture
        (ii) preparation of extract A
        (iii) preparation of extract B
      b) the packaging reaction
  - Quick analysis of phage DNA from a liquid lysate

VII  Gel electrophoresis
  - Agarose gels
  - Preparation of DNA fragments from agarose gels

VIII Plasmids
  - Preparation of plasmid DNA
  - Transfer of a plasmid
  - Plasmid transformation
  - Selection for restricting transformants
  - In vivo tests for restriction and modification
    1) bacteria
      a) restriction
b) modification  
2) phage  
a) restriction  
b) modification  
- In vitro test for PstI activity  
a) plasmid carrying cells  
b) induced cells  
c) infection experiments  
- Crude extract test for activity of HindIII, BamHI or SalI  

IX References
## I: Bacterial strains

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<td>HB101; recA&lt;sup&gt;R&lt;/sup&gt;rk&lt;sup&gt;-&lt;/sup&gt;mk&lt;sup&gt;-&lt;/sup&gt;pro&lt;sup&gt;-&lt;/sup&gt;leu&lt;sup&gt;-&lt;/sup&gt;thi&lt;sup&gt;-&lt;/sup&gt;lacY&lt;sup&gt;-&lt;/sup&gt; endA&lt;sup&gt;R&lt;/sup&gt;rps&lt;sub&gt;120&lt;/sub&gt; ara&lt;sub&gt;14&lt;/sub&gt; galK&lt;sub&gt;2&lt;/sub&gt; xyl&lt;sub&gt;5&lt;/sub&gt; mtl&lt;sub&gt;1&lt;/sub&gt; supE&lt;sup&gt;-&lt;/sup&gt;trp&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Boyer &amp; Roulland-Dussoix (1969)</td>
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<td>WL126</td>
<td>QR47((\lambda) WL34) = QR47((\lambda) supF&lt;sup&gt;-&lt;/sup&gt;imm21)</td>
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<tr>
<td>WL127</td>
<td>WL123 cured of prophage</td>
<td>this thesis</td>
</tr>
<tr>
<td>WL128</td>
<td>WL127(pWL98) amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>this thesis</td>
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<td>WL125(pWL98) amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>WL130</td>
<td>WL127(p(gam)) (gam&lt;sup&gt;+&lt;/sup&gt;kil&lt;sup&gt;+&lt;/sup&gt;cro&lt;sup&gt;-&lt;/sup&gt;); amp&lt;sup&gt;R&lt;/sup&gt;- Rec&lt;sup&gt;-&lt;/sup&gt; (\lambda)-immune, see text</td>
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<td>WL127(p(gam)); (gam&lt;sup&gt;+&lt;/sup&gt;kil&lt;sup&gt;+&lt;/sup&gt;cro&lt;sup&gt;-&lt;/sup&gt;); amp&lt;sup&gt;R&lt;/sup&gt; recA but not (\lambda)-immune, see text</td>
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<td>'WL125(p(gam))'; (gam&lt;sup&gt;+&lt;/sup&gt;kil&lt;sup&gt;+&lt;/sup&gt;cro&lt;sup&gt;-&lt;/sup&gt;); amp&lt;sup&gt;R&lt;/sup&gt; recA but not (\lambda)-immune, see text</td>
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<td>WL144</td>
<td>WL96(pAT153/Pst(120i)), isolates 1-8</td>
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<td>WL145</td>
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<td>WL146</td>
<td>large colony mutant at 42°C of WL136</td>
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<td>WL147</td>
<td>WL125(pWL98) ((\lambda) WL81), amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>WL148</td>
<td>WL149, amp&lt;sup&gt;S&lt;/sup&gt;</td>
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WL161 | AB1157 with known trp+ (OE1); T1R F- proA-his4 T6R SmR supE leu arg thr B1 lac ara - gal man xyl | Low (1973), L
WL162 | WL161(λ WL90) | this thesis
WL165 | WL127(p(gam)); ampR(gam kil cro+) Rec+, λ-immune, see text | this thesis
WL166 | λR WL98 | this thesis
WL167 | R WL96 | this thesis
WL169 | JC5088; Hfr recA56 specR SmS thr ilv | this thesis
WL170 | WL108(λ WL91); ampS; lost Pst-fragment | this thesis
WL182 | WL108(λ WL91 isol.1) isol.1; ampS see text | this thesis
WL183 | WL108(λ WL91 isol.1) isol.2; ampS see text | this thesis
WL184 | WL108(λ WL91 isol.2) isol.1; ampS see text | this thesis
WL185 | WL108(λ WL91 isol.2) isol.2; ampS see text | this thesis
WL186 | WL108(λ WL91 isol.3) isol.1; ampR R see text | this thesis
WL187 | WL108(λ WL91 isol.3) isol.4; ampR R see text | this thesis
WL188 | WL108(λ WL91 isol.3) isol.5; ampR see text | this thesis
WL189 | WL108(λ WL91 isol.4) isol.2; ampS see text | this thesis
WL190 | WL108(λ WL91 isol.5) isol.1; ampS see text | this thesis
WL191 | WL108(λ WL91 isol.5) isol.6; ampS see text | this thesis
WL192 | WL108(λ WL91 isol.6) isol.1; ampS see text | this thesis
WL193 | WL108(λ WL91 isol.6) isol.2; ampS see text | this thesis
WL194 | WL108(λ WL91 isol.7) isol.1; ampR see text | this thesis
WL195 | WL108(λ WL91 isol.7) isol.4; ampR see text | this thesis
WL196 | WL108(λ WL91 isol.8) isol.1; ampS see text | this thesis
WL197 | QR47(λ imm λ) | L
WL200 | WL46(λ WL103, isolates 2 and 3) (2002 and 2003) | this thesis
WL201 | WL161(λ WL1032) tonA (kil- lysogen) | this thesis
WL202 | WL161(λ WL1033) tonA (kil+ lysogen) | this thesis
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<td>WL217</td>
<td>WL30(pWL215)</td>
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* L = from the Leicester collection (prepared by W.J.Brammar, A.Smith, D.Burt, C.Hadfield, N.Grinter or myself).
### II: Phage strains

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<td>λWL8</td>
<td>λNM567; (sri λ1-2)(^v) sri λ3° imm434 cl(^-) srl λ4° srl λ5°</td>
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<td>Murray et al (1977)</td>
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<td>λWL11</td>
<td>h(^80) trp46 imm434 cl(^-) Pam80</td>
<td>R. Pastrana</td>
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<td>λWL12</td>
<td>λCH109; b538 imm434 cl(^-) sri λ4° Pam80 srl λ5°</td>
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<td>λWL17</td>
<td>λDB82; h(^80) att(^80) imm434 cl(^-) srl λ4° Pam80 srl λ5°</td>
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<td>λDB63; Jam6 bio1 cl(^857)</td>
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<td>λWL23</td>
<td>λWL1xλDB82; chiA131 lacZ cl(^-) srl λ4° Pam80-sri λ5°</td>
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<td>λWL24</td>
<td>λWJB15; h(^80) att λ cl(^-)</td>
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<td>λWL25</td>
<td>λWJB230; h(^80) att λ imm21 cl(^-)</td>
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<td>λWJB308; Eam4 Wam403 lacZ cl(^-) srl λ4°(nin5)(^v)- srl λ5°</td>
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<td>λL334; chiA131 red3 gam am210</td>
<td>Stahl et al (1975)</td>
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<td>λWL34</td>
<td>λWJB331; trpA(^1) imm21 (nin5)(^v) shn λ6°</td>
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<td>λWL37</td>
<td>lac/trp (red gam cl(^III))(^v) imm21 cIta srl λ4°- (nin5)(^v) shn λ6° srl λ5°</td>
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<td>λWL38</td>
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<td>λWL42</td>
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<td>( λWL41a+ λWL44 (BamHI)) sbhI λ 1°(srl λ 1-2)- srl λ 3° imm34 cl- srl λ 4°(nin5)- shn λ 6° srl λ 5° isolates 1, 6, 10, 11 &amp; 12, see text</td>
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<td>λWL52</td>
<td>Sam7 derivative of λWL47 by marker rescue</td>
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<td>λWL55</td>
<td>λWL47: E.coli (chi+) (BamHI) recombinant</td>
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<td>λWL47chi: E.coli (chi+) (BamHI) recombinant</td>
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<td>λWL57</td>
<td>λWL47chi6; sbhI λ 1° chi(srl λ 1-2)- srl λ 3°- imm434 cl- srl λ 4°(nin5)-shn λ 6° srl λ 5°</td>
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<td>λWL58</td>
<td>λWL57 Sam7; in vitro recombinant of λWL57 and λWL52 (EcoRI); sbhI λ 1° chi(srl λ 1-2)- srl λ 2- srlimm434) srl λ 3° imm434 cl- srl λ 4°(nin5)-shn λ 6° Sam7 srl λ 5°</td>
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<td>λWL62</td>
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<td>(\lambda WL83)</td>
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<td>(\lambda WL84)</td>
<td>(\lambda (sri \lambda 1-2))(\mu)mm21(nin5)(\mu)shn(\lambda 6^{o})(\lambda)NM540) grown on WL98; Pst -modified</td>
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<td>(\lambda) WL25 on WL98; Pst -modified</td>
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<td>(\lambda (sri \lambda 1-2))(\mu)pen sri (\lambda)3(^{+}) cI(\lambda)(857) sri (\lambda)4(^{o}(nin5))(\mu) - sri (\lambda)(5^{o})</td>
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<td>L376</td>
<td>EcoRI-generated inversion of (\lambda) WL41a</td>
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<td>L378</td>
<td>HindIII-generated inversion of (\lambda) WL41a</td>
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<td>L39°</td>
<td>(\lambda h^{30}) trpB72 cI^R57 (O82)^30</td>
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* L = strains from the Leicester collection

(DB strains are strains obtained from D. Burt. CH strains are strains obtained from C. Hadfield. WJB strains are strains obtained from W. J. Brammar.)

WL strains are strains prepared during the course of this work; \(\lambda\) WL37 was prepared by A. Smith.
All media and buffers were autoclaved for 15' at 15 lb/sq.in.

Luria broth (LB) (Lennox, 1955)
Bactotryptone 10 g/l; yeastextract 5 g/l; NaCl 5 g/l; glucose 1 g/l. 
Adjust to pH 7.0 with 5N NaOH and autoclave.
LB + Mg = LB + 10 mM MgSO₄.

BBL broth (BBL) (Parkinson, 1968)
BBL trypticase 10 g/l; NaCl 5 g/l; autoclave.

Minimal medium (MM)

use sterile solutions:
800 ml water; 200 ml 5x Spizizen salts; 10 ml 20% sucrose
or 970 ml water; 20 ml 50x VB salts; 10 ml 20% sucrose
and supplemented with necessary growth factors: amino acids 4 µg/ml
cas. acids 0.2%
vitamins 1 µg/ml

Spizizen salts (5x)
(NH₄)₂SO₄ 10 g/l; K₂HPO₄ 70 g/l; KH₂PO₄ 30 g/l; Na₃Citrate 5 g/l;
MgSO₄·7H₂O 1 g/l

VB salts (50x) (Vogel & Bonner, 1956)
MgSO₄·7H₂O 14.3 g/l; Citric acid·1H₂O 143 g/l; K₂HPO₄(anhydrous) 715 g/l;
NaNH₄HPO₄·4H₂O 250 g/l

L-agar (LA)
LB without glucose + 10 g/l Difco Bacto agar
BBL agar
BBL broth + 10 g/l agar

Minimal agar
300 ml water + 15 g/l agar; autoclave; add (sterile)
200 ml 5x Spizizen salts + 10 ml 20% sucrose
or 20 ml 50x VB-salts in 170 ml warm water + 10 ml 20% sucrose
(note: do not use sucrose if selecting for a sugar marker)

Top agar
BBL + 6.5 g/l agar; for BBL- and L-plates
water + 6.5 g/l agar; for MM-plates

Stab medium
LB + 0.65% agar; autoclave; add cys (20 μg/ml) and thy (40 μg/ml)
and fill up small screw cap glass vials (3/4 full); autoclave.

Cell buffer
KH₂PO₄  3 g/l; Na₂HPO₄ 7 g/l; NaCl 4 g/l; MgSO₄·7H₂O 0.2 g/l

Phage buffer
KH₂PO₄ 3 g/l; Na₂HPO₄ 7 g/l; NaCl 5 g/l; 1 M MgSO₄ 1 ml/l; 0.1 M CaCl₂
1 ml/l; 1% gelatine 1 ml/l.

Xg plates (Murray et al, 1977)
BBL-plates containing 20 μg/ml Xg
Xg = 5-bromo-4-chloro-3-indolyl-β-D-galactoside
A 2 mg/ml stock solution is made up in N,N-dimethylformamide and stored
at -20°C. If the response is low, use more Xg (50 μg/ml) in the plates
and/or an inducer IPTG (isopropyl-$\beta$-D-galactopyranoside)

Medium for Streptomyces albus and Bacillus amyloliquefaciens H
Both were grown in LB without supplements at 37°C.

Medium for Hemophilus influenzae Rd. (Smith & Wilcox, 1970)
Cells were grown at 37°C in Brain-heart infusion broth (Difco),
supplemented with 10 µg/ml hemin and 2 µg/ml NAD, which were filter-
sterilised and added to the autoclaved broth.
IV: Preparation of bacterial cells, tests and DNA preparation

Plating cells

Cells were grown till late log. phase, spun, washed once in equal volume 10 mM MgSO₄ and stored at 4°C in the same volume 10 mM MgSO₄. Most plating cells can be stored for several weeks, Rec⁻ strains (with the exception of QP48) and Pat-carrying strains have to be prepared fresh at least once a week.

Frozen cultures

Cells can be stored at -20°C. Resuspend 1 ml of a fresh ON in 1 ml 10 mM MgSO₄; add 2 ml 80% glycerol (in H₂O), mix and store in a screw-cap bottle.

Stabs

Cells can also be kept on stabs: inoculate a stab, incubate ON, wrap parafilm around the lid to prevent dehydration and store at room temperature.

Conjugation

a) check the Hfr by cross-streaking an early log. culture, resuspended in minimal medium, against the recipient on a selective plate. Growth on the intersection indicates a positive result.

b) dilute a fresh ON of donor and recipient 1:20 in LB and grow till OD₆₅₀=0.5. Mix 1 ml of each culture carefully in a 20 ml tube. Mate at 37°C with the tube at a 45° angle. Stop the mating at the desired time by vortexing vigorously for 1' and dilute 100 fold in MM. Plate dilutions on selective plates.

Test for UV-sensitivity

To test recombination-deficient strains for UV, drops of a fresh ON were run on L-plates and allowed to dry. Controls of known Rec⁺ and Rec⁻ hosts
were run on the same plates. The plates were irradiated with 0, 150 and 300 ergs/mm$^2$. Rec$^+$ hosts are resistant to 300 ergs/mm$^2$; recBC strains are slightly sensitive to 300 ergs/mm$^2$, while recA and recAD are sensitive to 150 ergs/mm$^2$.

Selection of cells resistant to $\phi 80$ or $\lambda$

a) to make a strain resistant to $\lambda (\lambda^R)$ or to $\phi 80$ (tonA), $h^\lambda c$ or $h^30 c$

was adsorbed to 0.1 ml plating cells at m.o.i. 5 and left shaking ON in 1 ml LB + Mg. The culture was streaked for survivors on L-plates. Single colonies were purified and checked for characteristics (see table M-I).

b) Alternatively one can spread phage ($10^9 \phi$/plate) on L-plates and plate serial dilutions of the culture on this lawn. Colonies are purified and checked as under a.

c) To select for cells resistant to both phages due to the presence of the Pst-genes (chapter 6) both $h^{30} c$ and $h^\lambda c$ were used with the method described under b.

Preparation of lysogens

a) Lysogens can be made by spotting a serial dilution of the phage onto the host strain on a L-plate. After incubation at $32^\circ C$ (for a ts mutant) or $37^\circ C$ the turbid centre is streaked on a L-plate. Single colonies are purified and checked (see table M-I).

b) lysogens can also be prepared using method b in the previous section.
Table M-I  Characteristics of resistant or lysogenic cells

Phages of two different immunities and both host ranges are spotted at $10^5$ to $10^7 \phi/ml$ on the strain to be tested and control strains.

(For convenience the behaviour of all 6 different phages is shown).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Testing phage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda$WL24</td>
</tr>
<tr>
<td>$\lambda^R$</td>
<td>+</td>
</tr>
<tr>
<td>tonA</td>
<td>-</td>
</tr>
<tr>
<td>($\lambda$)lysogen</td>
<td>-</td>
</tr>
<tr>
<td>($\lambda$imm21)lysogen</td>
<td>+</td>
</tr>
<tr>
<td>($\lambda$imm434)lysogen</td>
<td>+</td>
</tr>
</tbody>
</table>

$\lambda$WL24: $h^{800}_{\text{imm}}\lambda^c$; $\lambda$WL25: $h^{800}_{\text{imm}21c}\lambda$; $\lambda$WL37: $h^{\text{imm21}c}\text{Its}$;
$\lambda$WL38: $h^{800}_{\text{imm}434c}\lambda$; $\lambda$WL57: $h^{\text{imm}434c}\lambda$; $\lambda$WL73: $h^{\text{imm}^\lambda\lambda}c$

For simplification the abbreviations $h^{800}_c$ and $h^{\lambda}_c$ have been used if the hostrange was being tested; phages of at least two immunities were tested in such a case.

$\lambda^c$ is used in chapter 6 and can denote any of the six phage phenotypes; it is used when strains are checked for restriction or modification properties.

$\lambda^c_m$ denotes the same phages grown on WL98, modified against the PstI restriction activity.

Preparation of bacterial DNA (adapted from Marmur, 1961)

- Use a fresh ON or preferably a late log.culture.
- Spin, resuspend in sucrose-solution (1), 25 ml/3g cells;
- Add lysozyme-solution (2), 10 mg/3g cells; shake 80° at 37°C; place on ice
(till it starts to lyse, 5' for *E. coli*, longer for most other strains, especially ON cultures).
- Add half volume 0.25 M EDTA pH 8.0; leave on ice 5';
- Add one volume Triton-solution (3), leave on ice 20';
- Add 1/5 volume 5M NaClO₄;
- Shake 30' with one volume CIA (4);
- Spin 5' 2000 rpm, pipet off top-layer and add 2 volumes ethanol to this in a small beaker; wind the precipitating DNA around a glassrod and dissolve in half volume 0.1xSSC; make up to 1xSSC and shake 15' with equal volume CIA **;
Repeat from * till ** until little or no protein is left on the interphase; dissolve the DNA finally in a small volume (e.g. 2 ml) 0.1xSSC;
- Incubate 30' 37°C with RNAse (5) at a final concentration of 50 μg/ml;
- At this stage a pronase treatment can be included (see preparation phage DNA);
- Phenol extract twice;
- Dialyse against 10 mM Tris-HCl pH 8.0.

Solutions
(1) 25% sucrose in 50 mM Tris-HCl pH 8.0
(2) 10 mg/ml fresh stock solution in 250 mM Tris-HCl pH 8.0, 250 mM EDTA pH 8.0
(3) 2% Triton X-100 in 62.5 mM EDTA, 50 mM Tris-HCl pH 8.0
(4) Chloroform: isoamylalcohol 24:1
(5) 20 mg/ml RNase stock solution in 0.15 M NaCl pH 5.0, preheated 10' 80°C, stored at -20°C.
**Preparation of phages, tests and DNA preparation**

**Phage cross (Murray et al, 1973)**

The phages to be used for the cross were adsorbed onto a permissive host, usually QR47 or Ymel, for 20' at room temperature at m.o.i. 5.

The cells were diluted 100-fold in prewarmed LB + Mg and shaken 70' at 37°C. Dilutions were plated on selective hosts as well as control strains.

**Sam enrichment**

To enrich for the lysis-defective Sam marker in a cross, adsorb 0.1 ml of the cross mixture onto 0.5 ml non-suppressing plating cells and dilute into 20 ml LB + Mg. Grow 2 hrs. at 37°C. Pellet the cells and wash three times to get rid of lysis-proficient phage in the supernatant. Resuspend in 1 ml LB. Add chloroform to lyse the cells, spin and plate on a permissive host.

**Preparation of \( \lambda \) WL58 by marker rescue**

The 9.2% HindIII fragment containing Sam7 srI \( \lambda 5^0 \) was recovered from the DNA of \( \lambda \) WJB307 (chapter 3 section a) from a gel and used to transform \( \lambda \) WL47.1. After recovery from the gel and ethanol precipitation the DNA was resuspended in 0.1xSSC (100 μl). 100 μl competent cells were mixed with 50 μl 1xSSC and the DNA was added to this.

0.1 ml of this was mixed after transformation with 0.5 ml sup° cells and grown for 4 hrs. at 37°C. Cells were washed as above and plated on a suIII host. Individual plaques were tested for failure to grow on sup°. The left arm of this phage \( \lambda \) WL52 was replaced by one containing a chi-site by in vitro reconstitution of the left arm of \( \lambda \) WL57 with the central and right arms of \( \lambda \) WL52 (digested with EcoRI).

**Preparation of \( \lambda \) WL37**

This strain was prepared by Anne Smith in this laboratory by successive
cycling of λ DB64 (λ lacZ/ trp (red gam cIII) imm21 cIts srl λ 4°(nin5) shn λ 6° on an EcoRI-restricting host and a non-restricting host (5KRI (WL3) and 5K (WL2)). This method has been described by Murray & Murray (1974). λ WL3 subsequently carries a right arm devoid of targets for EcoRI, HindIII (and BamHI): srl λ 4°(nin5) shn λ 6° srl λ 5°.

Complementation test

Run drops of dilutions of the phage to be tested on a non-permissive host (at 10⁻⁷, 10⁻⁸ and 10⁻⁹/ml). When dried, run drops of known mutants cross-wise on the same plate. Growth on the intersection will indicate complementation.

LacZ test (Murray et al, 1977)

If the phage carries the whole lacZ gene of E.coli or that part of the gene carrying the lac-operator, the phage will titre out the repressor of lac in the cell during replication. Derepression of the gene can be visualised using a dye, Xg, in the plates. This dye changes from white to blue by the action of the β-galactosidase protein, the product of the lacZ gene. For this test a Lac⁺ host has to be used if the phage carries only part of the gene.

GroP test (Georgopoulos & Herskowitz, 1971)

To test a phage for carrying a mutation in the P gene, a groP host is used. P⁻-mutants grow on this strain whereas P⁺-phages fail on such a host, which has an altered dnaB product. The groP mutant is temperature-sensitive, the strain is grown at 32°C and used in a test at 37°C.

GroE test (Georgopoulos et al, 1973)

To test a phage for a mutation in the E gene, a groE host can be used. Mutant E⁻-phages grow on this host, E⁺-phages do not. The groE mutation is temperature-sensitive like groP; cells are therefore grown at 32°C and used at 37°C for a test.
**GroN test**

a) For $\text{(nin)}^\wedge$

A $\text{groN}$ host has an altered RNA polymerase, which can not be recognised by the $\lambda^N$-product. Transcription will therefore terminate at $t_{L1}$ and $t_{R1}$ and $t_{R2}$. The first two are both leaky, but $t_{R2}$ is a strong terminator. Late transcription will therefore only proceed if this terminator is removed (by a $\text{nin}$-deletion). Leaky transcription from the left strand through $t_{L1}$ will produce small amounts of $\text{red}$ and $\text{gam}$ leading to a small plaque phenotype of such phages on $\text{groN}$.

b) For $\text{chi(nin)}^\wedge$

Acquisition of a $\text{chi}$-site, which makes $\lambda$ DNA a good substrate for the host's recombination system, results in a large plaque phenotype. The presence of the $\text{chi}$-site has therefore usually been tested by testing the plaque size of that phage on $\text{groN}$ (see Anilionis, 1977).

Note: as discussed in chapter 3 this large plaque phenotype on $\text{groN}$ can also be due to $N$-independent $\text{red}$ and $\text{gam}$ expression.

**Supernus test** (Friedman et al, 1976)

Phage 21 has an $N$-product which will still recognise the altered RNA polymerase of the $\text{groN}$ host. Supernus is a host similar to $\text{groN}$, but in this case the phage 21 $N$-product fails to interact with this RNA polymerase while $\lambda^N$ works normally in such a host.

**Tests for the presence of the $\lambda$ red and gam genes**

The absence of the $\lambda$ $\text{red}$ and $\text{gam}$ genes can be tested by plating on a strain lysogenic for phage P2. Only $\lambda^\text{red}^\text{gam}^-$ phage can grow on such a host due to the action of the P2-old product which is produced in such a lysogen and inhibits growth of $\lambda^+$. ($\text{Spi}$-phenotype; s.p.i.: susceptibility to prophage inhibition). Again, such phages will grow poorly, unless they carry a $\text{chi}$-site (full $\text{Spi}$-phenotype).
The presence of these genes can also be tested on a recA host. $\lambda^+$ can grow on such a strain, but $\lambda red^- gam^-$ can not, unless it carries the bacterial gene sbcA (fig I-4). Such a $\lambda sbcA$ phage is called $\lambda$ 'reverse' (Gottesmann et al, 1974). Failure to grow on recA is called the Fec- phenotype.

Phages deficient in red or gam or both fail to plate on polA or lig ts hosts. (See Introduction). This is called the Feb- phenotype.

These tests are summarised in table M-2.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Host</th>
<th>Rec+</th>
<th>recA</th>
<th>Rec+(P2)</th>
<th>polA/lig ts</th>
<th>phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda red^- gam^-$</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Spi-Fec-Feb-</td>
</tr>
<tr>
<td>$\lambda red^+ gam^+$</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Spi+Fec+Feb+</td>
</tr>
<tr>
<td>$\lambda red^-$</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Spi+Fec-Feb-</td>
</tr>
<tr>
<td>$\lambda gam^-$</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Spi+Fec+Feb-</td>
</tr>
</tbody>
</table>

standard hosts: C600 (WL1) or QR47 (WL11) for Rec+; QR48 (WL30) for recA; sup°(P2)(WL12) or C600(P2) (WL60); lig ts7 supF for the Feb-test (WL35).

Test for the number of EcoRI targets in $\lambda$

To determine the number of restriction targets for EcoRI in the phages from the crosses in chapters 1 and 3, the e.o.p. was compared of that phage on a non-restricting host (5K (WL2)) and the same strain carrying a plasmid with the EcoRI genes for restriction and modification, 5KRI (WL3). One target for the enzyme will reduce the e.o.p. by $10^{-1}$, two sites $10^{-2}$ etc.

This restriction ratio was checked in all crosses of chapters 1 and 3 where applicable.
Phage preparations

Small amounts of phage are prepared from plate lysates or 10 ml blattners; large amounts (for DNA preparations) from 200 ml lysates in liquid medium. Blattners can only be used for the preparation of clear phages.

a) Platelysates

Resuspend a fresh plaque in 1 ml phage buffer + a drop of chloroform.

Allow to resuspend for 10'. Adsorb 0.1 ml of the phage suspension onto 0.1 ml fresh plating cells, 10' at rT. Add 3 ml top agar and plate on fresh wet L-plates. Incubate for 5-8 hours at 37°C (or 32°C). Add 4 ml LB to the plate and harvest broth and top-layer. Add a drop of chloroform, spin and titre the supernatant. Store the phage preparation at 4°C in a bottle, which has to be free of detergent.

b) 'Blattner'

The original method described by Blattner et al (1977) as the preadsorb-dilute-shake method (called a 'blattner' in our lab) uses 0.1 ml of a fresh phage suspension, prepared from a single plaque as under a . After adsorption for 10' at rT to 0.1 ml of fresh plating cells, the cells are diluted into 200 ml LB + Mg in a 2 l flask and shaken ON at 37°C.

After the culture has lysed, chloroform (2 ml/l) is added to release the phage from unlysed cells, spun and treated as under a .

This method often does not work with small or other slow-growing phages, because the original phage-input is too low. It also does not always work with less efficient host strains (e.g. Rec-).

I therefore decided to put a whole plaque in 200 ml LB + Mg. This method proved to work well with most phages and host strains.

The best results have been obtained by plating for single plaques early in the morning and transferring a whole plaque, or (if the plaques are small) several plaques, into 200 ml LB + Mg. After ON incubation the culture usually has lysed. If little or no lysis has occurred, good yields can often be obtained by diluting the culture 1:4 in fresh LB + Mg and longer incu-
This will lead to lysis in 3 to 4 hours, sometimes after ON incubation, unless the original phage-input was far too low or the original number of bacteria far too high. The first will happen with slow-growing phages, as already mentioned above and can be solved by using more plaques (e.g. up to 10 plaques have been used for very small phages), the second when using cells which grow too fast, e.g. w3350. By using the end of a pasteur pipet rather than a plaque-picker (which is much narrower) we can reduce the input of bacteria without increasing the number of phage. A shaker at 150 rpm with normal (i.e. non-baffled) flasks has given me the best results.

c) Liquid lysate

This method can be used for the preparation of clear phages, but is the only method for large-scale preparation of turbid phages.

Early log-phase cells are infected with phage at m.o.i. 5. and allowed one single burst.

Prepare a phage stock using method a .

Dilute a fresh ON of a permissive host 1:20 in 200 ml LB + Mg in a 2 l flask. Grow till OD$^{650} = 0.5$ and add phage (m.o.i. 5). Grow with vigorous aeration, monitor the OD. This will first rise, then start to fall when the cells begin to lyse, then rise again. Harvest the cells as soon as the OD goes up the second time.

Preparation of phage DNA

Use 200-1000 ml of $2\times10^9$ to $10^{11}$ $\phi$/ml lysates, prepared as described above. Concentrate the phage by pelleting or PEG-precipitation, before purification on a Cs-gradient.
1) Concentration of the phage

a) pelleting of the phage by centrifugation

Spin a cleared lysate 3 hrs at 21K. Resuspend the pellet ON by shaking very gently in a small volume at 4°C. Spin 10' 10K to remove bacterial debris. Treat the supernatant with 10 μg/ml DNase and RNase. Leave 1 hr at rT. If a clean preparation is required, repellet the phage 3 hrs at 26 K (10 x 10, MSC65) and resuspend ON. Respin 10' 10K and band the phage on a Cs-gradient.

b) PEG-precipitation (Yamamoto et al, 1970)

Make a cleared lysate up to 0.5 M NaCl using solid salt. Add 1 μg/ml DNase and RNase (B-grade) and leave 1 hr at rT. Add PEG-6000 (10% w/v) and dissolve at rT. Leave on ice for 1 hr (or ON at 4°C). Spin 5' 2000 rpm and resuspend the pellet very gently in 1/20 - 1/50 of the original volume in phage buffer.

2) Purification of the concentrated phage on a Cs-gradient

One can either use a step gradient or a continuous gradient, spun to equilibrium. When very clean DNA preparations are desired, phage can first be banded on a step-gradient, followed by a second purification step using the second method.

a) CsCl step gradient (adapted from Miller, 1972)

Three Cs-layers are used at 1.7, 1.5 and 1.3 g/ml. A maximum of 7 ml phage suspension can be loaded on such a gradient.

Preparation of Cs-solutions:

Prepare a stock solution of saturated CsCl in water (65 g CsCl + 35 g H₂O) at rT. Dilute with phage buffer as follows:

(i) 1.7 g/ml: 4.66 ml CsCl + 2 ml phage buffer
(ii) 1.5 g/ml: 2.22 ml CsCl + 1.78 ml phage buffer
(iii) 1.3 g/ml: 1.33 ml CsCl + 2.11 ml phage buffer
Pipet 3 ml solution (iii) in a 14 ml centrifuge tube.
Layer with a long pasteur pipet 2 ml solution (ii) underneath, followed by 2 ml (i).
Layer the phage preparation on top of the gradient.
Spin 1-2 hrs at 35K at 20°C in the 6 x 14 ml swing-out rotor.
Remove the phage band using a syringe, piercing the needle through the side of the tube (phage can thus be stored for years at 4°C).
If the phage lysate was old, or not handled carefully during resuspension, a second band will be visible between the 1.5 and 1.7 layers, which does not contain DNA.

b) CsCl equilibrium gradient
Add 0.71 g CsCl/g phage solution. Make up to volume with 41.5% CsCl in phage buffer. Spin 24-36 hrs at 33K (4°C) in a 6 x 14 ml swing-out rotor. Remove the band using a syringe as above.

3) Preparation of the DNA
Dialyse the phage in CsCl against TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) at 4°C (minimal 1 hr).
To reduce the number of single-stranded breaks in the DNA the phage can be treated with pronase (this is advisable when transfecting).
a) pronase treatment
A pronase stock solution is prepared at 10 mg/ml and preincubated 1 hr at 37°C (autodigestion) before use. It can be stored at -20°C.
Put the phage in a plastic tube and add pronase at a final concentration of 1 mg/ml. Dialyse 2 hrs at 37°C against pronase buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.002% Triton X-100).
Upon subsequent phenol extraction phenol equilibrated with this buffer instead of TE and dialyse afterwards against 20 mM Tris-HCl pH 7.5, 0.1 mM EDTA.
b) phenol extraction
Redistil solid phenol under N₂ and store at 4°C under oxygen-free water.
Phenol can thus be kept for 3 months. It can also be stored frozen at 
-20°C after saturation with 0.5 M Tris-HCl pH 3.0.

Before use the phenol is pre-equilibrated with 0.5 M Tris-HCl pH 8.0  
(or pronase buffer).

Add phenol to the phage (1:1 v/v) and roll gently for 5' at rT. Spin  
at low speed. Remove the phenol-layer with a pasteur pipet. Repeat twice  
or until both layers are clear and there is no protein visible on the  
interphase.

Dialyse the DNA (top-layer) against TE at 4°C, 4 changes in 24 hrs.  
(Dialysis tubing 8/32, boil 20' in 1 mM EDTA before use).

c) DNA content

Determine the DNA concentration of the preparation by reading the OD_{260}.

OD_{260} = 1 is equivalent to 50 μg/ml. Usually a 1/10 or 1/100 dilution  
was measured and discarded. The ratio OD_{260/280} should be 1.8-2.0 for  
a clean DNA preparation.
VI: In vitro manipulations

Restriction

The nomenclature for restriction enzymes is as proposed by Smith & Nathans (1973). For targets of these enzymes I have used the simplified abbreviation for the enzyme followed by the genome concerned (EcoRI sites in λ : srI λ1 etc; BamHI sites in λ : sbhl λ1 etc or a further simplification for shindIII to shn). In the figures mutations removing relevant targets have been simply notated as a number (e.g. 3° for srI λ3°). The full notation can be found in the accompanying text.

a) Restriction reaction

For gel analysis 0.5 µg DNA was digested in a small volume (20-40 µl) with 0.5 unit of enzyme in the appropriate restriction buffer (1 hr at 37°C).

The reaction was stopped by 10' incubation at 70°C.

2x and 10x stock solutions of the restriction buffers were stored at 4°C.

When double digests were carried out, the buffer containing the lowest salt concentration was used. If digests could not be carried out simultaneously DNA was restricted with one enzyme, precipitated with ethanol and digested with the second enzyme.

Restriction assay buffers:

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tris-HCl (pH)</th>
<th>NaCl</th>
<th>MgCl₂</th>
<th>2-ME</th>
<th>DTT</th>
<th>(NH₄)₂SO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>BamHI</td>
<td>6</td>
<td>50</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EcoRI</td>
<td>100</td>
<td>50</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HindIII</td>
<td>60</td>
<td>7</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>or</td>
<td>7</td>
<td>60</td>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KpnI</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MboI</td>
<td>10</td>
<td>75</td>
<td>10</td>
<td>-</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>PstI</td>
<td>20</td>
<td>10</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>SauI</td>
<td>6</td>
<td>75</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XhoI</td>
<td>6</td>
<td>75</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
All concentrations in mM;
2-ME: \( \gamma \)-mercaptoethanol;
DTT: dithiothreitol

b) Ethanol precipitation
To clean or concentrate the DNA or change the buffer, the DNA can be
ethanol precipitated.
Add 1/10 volume 2 M NaAc pH 5.6 to the DNA in an eppendorf tube.
Add 2 volumes of cold (-20°C) ethanol. Leave ON at -20°C, 1 hr at -70°C
or 15' in a dry ice/ethanol bath.
Spin 15' in the Eppendorf centrifuge, pour off the supernatant and dry
the pellet. Resuspend in TE.

c) Isopropanol treatment
Sometimes the DNA can not be digested. Isopropanol treatment often
solves this problem. Add 1/10 volume 2 M NaAc pH 5.6 to the DNA and
0.54 ml isopropanol per ml DNA. Leave ON at -20°C, 1 hr at -70°C or
15' in a dry ice/ethanol bath. Spin and resuspend in TE.

d) Methoxyethanol extraction
Another cleaning method is treatment with methoxyethanol.
Add to 1 volume DNA 1 volume 2.5 M KPO\(_4\) pH 8.0. Add 2 volumes of methoxy-
ethanol to this. Spin and remove the toplayer (DNA). Reextract the
bottom layer with 1 volume water and 1 volume KPO\(_4\) pH 8.0. Pool the
top-layers and precipitate with ethanol.

Ligation
The number of recombinants needed in order to find a given gene is dependent
on the genome size and the average length of the cloned fragments:
\[
N = \frac{\ln(1-x)}{\ln(1-f)}, \text{ in which } N = \text{number of recombinants required}
\]
\[
x = \text{probability to find a given gene}
\]
\[
f = \text{fraction of the genome represented by the}
\text{average target molecule size}
\]
The number of recombinants e.g. needed to find a given gene in *E. coli* (5.10^6 bp) with 99% probability, will be, if we assume an average target size of 20 kb: \( \frac{\ln(1-0.99)}{\ln(1-2.10^5/5.10^6)} = 1176 \) clones. Likewise for an average target size of 10 kb, ca. 2000 clones. The values for various fragments are plotted for *E. coli* in the following graph (fig M-1):

**Fig. M-I: Number of recombinants required to find a given *E. coli* gene with 99% probability at varying target length.**

\[ N = \frac{\ln(1-x)}{\ln(1-0.99)} \times \frac{1}{5.10^6} \]

\[ N = \text{number of recombinants required} \]
\[ t = \text{average target molecule length (in kb)} \]

The concentration of the DNA in the ligation mixture for optimal conditions is dependent on the length of vector and target fragments.
For \( \lambda \) the following formula can be used (Blattner, 1978):

\[
C = 46 \frac{V}{L^{3/2}}
\]

in which \( C \) = concentration to be used for the vector for optimal ligation

\( V \) = vector length in \% of \( \lambda^+ \) length

\( L \) = length of average target molecule in kb.

To clone 18 kb fragments into \( \lambda WL57 \) (82\%) the vector concentration to be used is \( 46.32 \times (18)^{3/2} \) = ca. 50 \( \mu \)g/ml. Choosing a molar ratio of vector: target of 1:1, we need a target concentration of \( 37/82 \times 50 \mu \)g/ml = 22 \( \mu \)g/ml, (18 kb = 37\%). Using a molar ratio of 1:1 the concentrations of vector and target DNA for cloning into \( \lambda WL57 \) are plotted in the next graph (fig M-2):

**Fig. M-2:** Optimal concentrations for ligation using \( \lambda WL57 \) as vector with varying target length.

\( c_\lambda = \) vector DNA concentration to be used.

\( c_t = \) target DNA concentration to be used.
a) Ligation reaction

Ligations were normally carried out in a 20-60 ul volume at a final concentration of 50 µg/ml (originally 30 µg/ml). When optimal conditions were needed the above formula was used. T₄-DNA ligase was prepared as described by Knopf (1977) from NM1100 (WL123) (Murray et al, 1979). The vector arms can be annealed before ligation: precipitate the DNA after restriction and resuspend in 10 mM Tris pH 7.5; add 2 µl 1 M Tris-HCl pH 7.5 and 1 µl 0.2 M MgCl₂; leave 1 hr at 4°C.

The DNA was made up to the desired volume with 10 mM Tris-HCl pH 7.5, 100 mM NaCl and 1/10 volume of a 10x ligase 'cocktail' buffer was added. The 'cocktail' was either freshly prepared (for optimal conditions) or defrosted (it was never defrosted more than 3x since it contains ATP).

Ligase 'cocktail':

660 mM Tris-HCl pH 7.5, 10 mM EDTA pH 9.0, 100 mM MgCl₂, 100 mM 2-ME, 10 mM ATP, 400 mM NaCl.

Ligations were incubated ON at 12°C.

The ligated DNA was either used for transformation or packaged in vitro.

Phage recovery after ligation

1) Transfection with λ DNA

a) competent cells for transformation (adapted from Mandel & Higa, 1970)

Dilute a fresh ON of a competent strain (WL65) 1:50 in LB and grow till OD₆₅₀ = 0.55 (or dilute 1:25 and grow 100' (37°C)). Chill the culture on ice, leave 45'. Spin the cells, wash in equal volume cold 100 mM MgCl₂, spin, wash in half volume 100 mM MgCl₂, spin and resuspend in 1/10 volume cold 100 mM CaCl₂. Keep on ice till use.

Note: for transfection with phage acid-washed glassware is necessary.

b) transformation

For transformation 20-30 µl DNA was mixed with 100-200 µl competent
cells on ice. After 30' on ice the cells were given a heat-shock at 42°C for 2' and put back on ice. 10 and 50 µl samples were mixed with 100 µplating cells and plated on BBL-plates.

2) In vitro packaging of λDNA (Sternberg et al, 1977)

a) preparation of in vitro packaging extracts

(i) induction of the cultures
- dilute a fresh ON of WL51 1:100 in 150 ml LB + Mg in a 2 l flask
  and dilute a fresh ON of WL52 1:100 in 750 ml LB + Mg in a 2 l baffled flask.
  Shake vigorously at 32°C for 3-4 hrs till OD₆₃₀ = 0.2.
- induce the cultures by shaking them in a 90°C water bath; monitor
  the temperature in the flask with an alcohol sterilised thermometer
  till it is 42°C.
- incubate under vigorous aeration 20' at 42°C then shift the
  temperature to 38°C for another 70'.
- chill the cultures in an ice-water bath.

(ii) preparation of extract A
Mix 150 ml of both cultures:
- spin and resuspend in 600 µl buffer A in a polypropylene tube and
  take a 50 µl sample.
- sonicate 12x2'' at 30'' intervals (amplitude 10-12 u) and take a
  50 µl sample.
- spin the two samples: little or no pellet in the second tube as
  compared to the first indicates proper sonication.
- divide into 50 µl samples in cold screw-cap vials and submerge in
  liquid N₂.
- store at -70°C.

(iii) preparation of extract B
- spin the remaining 600 ml of WL52.
- dry the pellet with a tissue and resuspend in 1.2 ml sucrose-buffer.
- divide into 80 µl aliquots in cold screw-cap vials and submerge in liquid N₂.
- thaw 10-12 tubes at a time in water at rT, freeze again and thaw.
- add 4 µl lysozyme-solution and stir with a 10 µl micropipet; incubate 30' on ice.
- mix in a separate tube on ice 625 µl glycerol and 200 µl buffer B.
- add 33 µl of this to each tube, stir, freeze in liquid N₂ and store at -70°C.

b) the packaging reaction

- mix at rT 30 µl buffer A + 2 µl buffer B + 20 µl extract A + 5 µl DNA (5-50 µg/ml), incubate 15' at rT.
- add 100 µl extract B stir and incubate 60' at 37°C.
- add 50 µl DNAse buffer and incubate 15' at 37°C, stirring occasionally with a 10 µl micropipet.
- add a drop of chloroform, spin and plate with fresh plating cells, grown in LB + Mg + 0.2% maltose instead of glucose.

Solutions:

Buffer A : 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 3 mM MgCl₂, 5 mM 2-ME
Buffer B : 6 mM Tris-HCl pH 7.4, 15 mM ATP, 16 mM MgCl₂, 30 mM 2-ME, 60 mM spermidine-HCl
Sucrose buffer: 50 mM Tris-HCl pH 7.4, 10% sucrose
Lysozyme solution: 1 mg/ml egg white lysozyme in 0.25 M Tris-HCl pH 7.4
DNAse buffer: 100 mM NaCl, 10 mM MgSO₄, 50 mM Tris-HCl pH 7.5, 0.01% gelatin, 10 µg/ml DNAse I

Recombinant phages can only be plated directly on C600 or C600(P2), if the cloned DNA is K-modified. Otherwise phages must be plated on a rk⁻mk⁺ host (WL65 or WL2).
The efficiency of the in vitro packaging procedure is $2-10 \times 10^7 \phi/\mu g$ DNA with a 10-100 fold reduction when religated DNA is used (Sternberg et al, 1977). Typical efficiencies in my hands were $4-10 \times 10^5 \phi/\mu g$ DNA.

**Rapid preparation of phage DNA for gel analysis**

(Blattner, 1978, adapted from Cameron et al, 1977)

- take 2 ml of a clarified liquid lysate or plate lysate (prepared on agarose plates instead of agar).
- add 0.4 ml SDS-mix and heat 30' at 65°C.
- chill 15' on ice.
- spin 15' 10000 rpm.
- add 5.6 ml ethanol to the supernatant, mix and leave 1 hr at -20°C or 20' at -70°C.
- spin 30' 17000 rpm.
- decant, dry pellet and dissolve in 0.4 ml 2 M NH$_4$Ac, transfer into an eppendorf tube.
- add 800 µl ethanol, mix and chill briefly at -20°C.
- spin 5' in the eppendorf centrifuge.
- decant, dry pellet and resuspend in 50 µl 1 mM EDTA pH 8.0.
- digest 1-1.5 µl of this preparation in a 10 µl volume.

SDS-mix: 0.25 M EDTA, 0.5 M Tris-HCl pH 9.0, 2.5% SDS

Note: results have been variable using this method; sometimes there is no digestion at all, sometimes all DNA is attacked by nucleases, sometimes beautiful digests are obtained.

The method can be scaled down 10-fold, starting with 200 µl and carrying out all pelleting steps in the eppendorf centrifuge.
VII: Gel electrophoresis

Agarose gels (Murray et al, 1977)

For gel analysis of restriction digest 0.7\% agarose gels (150 ml) were run ON at 20 mA in the horizontal slab gel set-up (see e.g. C.Hadfield or D.Burt Ph.D Theses) using paper wicks (6 layers of Whatman filter paper no 1) to connect the gel with the gel buffer.

Agarose was dissolved by heating, cooled till 50°C and poured. Usually Ethidium bromide was added to the gel (10 µg/ml) before pouring and to the buffer.

For the preparation of fragments from a gel 0.5\% gels were run for 2-4 hrs at 200 mA in the 'concorde' set-up in which the gel is submerged in the buffer. The buffer used in this case is half the normal strength to increase the speed. For a good resolution thick gels were used (300 ml). Ethidium bromide was added at 10 µg/ml and gels were run in the dark or covered with aluminium foil.

Gel buffer: 40 mM Tris, 20 mM NaAc, 1 mM EDTA; pH 8.2

Gel loading buffer: 10 mM Tris-HCl pH 7.5, 20 mM EDTA, 10\% glycerol, 0.01\% bromophenol blue, 20 mg agarose. Heat to dissolve the agarose and allow to set. Force the buffer through a hypodermic needle to make a fine slurry ('Agarose beads'; this buffer reduces trailing); store at 4°C.

Photography of gels: gels were viewed under UV and photographed using a red filter (Kodak wratten filter no 9) and polaroid film.

Preparation of DNA fragments from agarose gels

Run a gel as described in the previous section, in the dark. Use a long-wave UV lamp to visualise the bands. Cut out the desired bands with a scalpel.

Transfer the fragment into a dialysis bag (18/32 tubing) with gel buffer and
electro-elute till the DNA has left the agarose (20-30' for a small fragment up to hours for large ones). Reverse the current for a short period (10-15'') to release DNA bound to the dialysis membrane and transfer the buffer into a plastic centrifuge tube with screw-cap. Extract the DNA with isobutanol to remove the ethidium bromide. Further handling can take place in the light. Repeat the iso-butanol extraction to reduce the volume to 400 µl. Pass the DNA through a column and precipitate the DNA. Resuspend in TE. Yields are variable especially with large fragments (20-80%). DNA prepared in this way is restrictable and ligatable. For marker rescue the column step can be eliminated. Column:
a) Pour a 250-500 µl column in a blue or yellow gilson tip, which contains siliconised glasswool in the tip. Either G50 or DEAD52 can be used. I used the second. The principle is that the DNA will be retained on the column at low salt concentration (such as TE). Washing the column with high salt elutes the DNA.
b) Wash the column thoroughly after pouring with TE, load the DNA, wash the column with TE (3 volumes) and elute the DNA with TE containing 2 M NaCl (or 2 M NaCl, 50 mM Tris-HCl pH 8.0, 1 mM EDTA), 2 x 50 µl. The end volume will be about 200 µl. Precipitate with ethanol.

Note: There are various ways of cleaning the DNA after electro-elution. Another method is phenol extraction after the iso-butanol step, followed by iso-butanol and ether extractions to remove the phenol and the iso-butanol. Some people electro-elute in a 400 µl volume and do not treat the preparation with iso-butanol first, but extract with phenol immediately.
VIII: Plasmids

Preparation of plasmid DNA (Windass, pers.comm.)

Grow 100-200 ml cells ON or till late log.phase (OD\textsubscript{650} = 1-2)
- spin, resuspend in 2.4 ml 25% sucrose;
- add 0.4 ml 10 mg/ml lysozyme (in water) leave on ice for 5';
- add 0.8 ml 0.25 M EDTA pH 8.0 and 3.2 ml 2% Triton X-100, leave on ice till lysed;
- tip into a 50 or 100 ml tube and spin 30' 18K, do not use the brake;
- decant and make the supernatant up to 6.56 ml with 25% sucrose, 0.05 M EDTA;
- add 6.32 g CsCl and 240 \mu l ethidium bromide (10 mg/ml) and spin 40 hrs 40K (20°C) in the 10x10 Ti rotor;
- remove the plasmid band in the dark under long-wave UV with a syringe;
- saturate a TE solution with CsCl and saturate iso-propanol with this;
- mix the plasmid preparation 1:1 with the iso-propanol to remove the ethidium bromide and dialyse to remove the CsCl.

Originally preparations were made like this, however, the DNA appears to contain nucleases since upon prolonged storage at 4°C it tends to get degraded (e.g. DNA of pWL98). For long term storage phenol extraction is necessary. All later plasmid preparations were therefore treated with phenol (followed by iso-butanol/ether and ethanol precipitation steps). For transformation phenol extraction is not necessary (see next section).

Transfer of a plasmid

To transfer a plasmid from one strain into another, a cleared lysate can be used (e.g. to transfer pWL136 into a recA background). A cleared lysate was made of a 2 ml ON culture as described above. Instead of a spin of 30' at 18K the lysate was cleared by a 15' spin in the eppendorf centrifuge. The DNA in the cleared lysate was precipitated with ethanol (lysate + 10 \mu l 2 M NaAc + 200 \mu l ethanol, 20'-70°C) and the pellet resuspended in 20 \mu l 0.1 x SSC; 2 \mu l
1 x SSC and 44 μl ethanol were added for a second precipitation step. The pellet was resuspended in 100 μl 0.1 x SSC and mixed with 200 μl competent cells of the recipient strain.

**Plasmid transformation**

The cells were made competent as described for phage transfection. Acid-washed glassware is not necessary. Cells were not kept on ice 45' prior to their first spin. Usually WL96(Rec+) & WL125(recA) were used as hosts. 10-20 μl DNA was mixed with 100 μl competent cells; after a heatshock at 42°C the cells were left on ice for 1 hr. 1 ml LB was added and the cells incubated at 32°C for 30-60' before selection for ampicillin resistance.

Ampicillin was used at 30 μg/ml in LB, BBL- or L-plates; after transformation 100 μg/ml was used if the culture was further challenged with phage. Tetracycline resistance was checked using 20 μg/ml in media or plates.

**Selection for restricting transformants (Pst, chapter 6)**

To select for transformed cells carrying the Pst-genes for restriction and modification, cells were first grown in LB+amp, with fresh amp added after 2 hrs. Cells were spun down and resuspended in 1 ml LB+Mg+amp. Control samples were plated on L- and L+amp-plates. The remainder was challenged with phage. 1, 10, 50 and 100 μl cells were incubated on L+amp plates on which two phages with different host range and immunity were spread (10^9 φ/plate each) (usually combinations of λWL24+λWL57 or λWL73+λWL25 were used). Surviving cells included cell wall mutants (resistant to both phages), cells which had escaped infection and the desired recombinants.

**In vivo tests for restriction and modification**

1) Bacteria
   a) restriction

   To check for restriction phages of both host ranges and at least two
immunities were used (usual combination: \( \lambda \) WL73 + \( \lambda \) WL24 + \( \lambda \) WL57 + \( \lambda \) WL25). Once WL98 was constructed, modified lysates of these phages were prepared and used as controls (\( \lambda \) WL75, \( \lambda \) WL79, \( \lambda \) WL78 and \( \lambda \) WL104 respectively).

Modified phage is indicated by \( \lambda c_m \).

(A strain restricts if it allows a modified phage, but not its unmodified version to plate at normal efficiencies).

b) modification

To check a strain for modification a plaque of one of the four phages mentioned above was plated on the strain and spot tested on C600 and WL98. If the e.o.p. is high, the strain carries the modification gene. If the e.o.p. is low (usually this means no plaques on WL98), the modification activity is absent.

In the case of a lysogen, the induced phage was checked on C600 and WL98. If the lysogen expresses the modification gene, it will produce plaques on both hosts, when we simply spot a drop of the culture on a bacterial lawn of C600 or WL98.

In the case of WL154, a \( \lambda^{80} \) phage was spotted on the lysogen and subsequently tested on \( \lambda^R \) derivatives of WL96 and WL98, which allowed determination of the exact level of modification.

2) Phage

a) restriction

To check a phage for its ability to restrict in vivo, crosses were carried out on QR47 between this phage and another phage, which had been grown on QR47 (unmodified) and WL98 (modified). If the phage expresses the restriction gene, the number of recombinants in the first cross should be substantially lower than in the second cross. These crosses were therefore plated on a host, which was permissive for recombinants only.
b) modification

To check a phage for its ability to modify its own DNA, a plaque of this phage on C600 was spot tested on WL98 and C600 (or WL96). No plaques on WL98 indicates lack of modification (Note: the DNA is only partially modified, so the e.o.p. is < 1, see chapter 6).

**In vitro test for PstI activity (crude extract test)**

a) plasmid carrying cells (WL98 (etc.) at 37°C and WL108 (etc.) at 32°C)

Dilute a fresh ON 1:25 in 100 ml LB+amp and grow till late log.phase.
- spin and resuspend in 1 ml PstI assay buffer (cold);
- sonicate 3 x 20'' at 1' intervals (maximum output), keeping the tube on an ice-water bath;
- transfer into a cold eppendorf tube and spin 10' (4°C);
- pipet off the supernatant and prepare dilutions on ice in PstI assay buffer;
- digest 0.2 ug λ+DNA with 2 μl extract and dilutions thereof for 20', 40' and 60' at 37°C.
- heatkill the reaction and analyse the restriction pattern on a gel.

b) induced cells (lysogens and derivatives of pL-asmid)

Dilute a fresh ON 1:25 in fresh medium and grow till OD$_{650}$ = 0.3 - 0.4 at 32°C.
- induce the culture at 42°C in a 90°C water-bath;
- grow at 39°C for 1-2 hrs and assay as under a.

An uninduced control was grown for the same length of time at 32°C, as well as a WL98 control (at 37°C).

c) infection experiments

Phages expressing *in vivo* modification but no restriction were checked in an *in vitro* test for restriction: C600 cells were grown till OD$_{650}$ = 0.5 and infected at m.o.i. 5; after an additional 1 hr growth cells were harvested and a crude extract assay carried out as described above.
Crude extract test for activity of HindIII, SalI and BamHI

These were carried out as for the activity of the PsiI restriction enzyme, substituting the assay buffer for those of the appropriate enzyme.
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Results

Chapter 1

Preparation of a λ vector for cloning large restriction fragments made by digestion with EcoRI and HindIII.

The phage vector λNM567, λ(srl λ1-2) srl λ30 imm434 cl srl λ40(nin5) srl λ50, was designed as a large capacity cloning vector for use with EcoRI (fig. 1-1). It is a replacement vector using the hybrid EcoRI site srl λ1/2 and srl (imm434) flanking a 10.6 kb central fragment. Selection of recombinants is based upon the disappearance of this central fragment, containing the red and gam genes, generating the Spi− phenotype (M-20).

The minimum insert size is 8.6 kb, the maximum capacity 24 kb. However, recombinants based on this vector will grow poorly unless they carry a chi-sequence on the cloned fragment (I-15).

Fig. 1-1: λ NM567λ L334

\[
\begin{align*}
\text{R} & \quad \text{3°} & \quad \text{4°(nin5)} & \quad \text{5°} \\
\lambda \text{NM567} & \quad \chi \text{A} & \quad \text{red gamam} & \quad \lambda \text{L334}
\end{align*}
\]

To overcome this problem of poor growth of recombinants based on the vector λNM567, a chi-site was introduced into the left arm of the vector. The phage should then be suitable for cloning DNA from any source and not just DNA carrying (a) chi-sequence(s).

To make this vector suitable for cloning with restriction enzyme HindIII as well, a shn λ60 mutation was subsequently introduced into the right arm of
this phage, λWL28, to produce the EcoRI - HindIII vector λWL41.

a) Introduction of chiA into λNM567.

The chiA mutation lies in the λ gene at about 36% on the λ map (Stahl et al., 1980). As source for the chi mutation the following phage was used: h² chiA131-red3 gam am210 (see fig 1-1; λL334= λWL27). If we cross this phage with λNM567, we cannot easily find the desired recombinant. Apart from the danger of multiple crossover events, which might reintroduce targets for EcoRI from the λL334 parent into λNM567, selection is difficult. The parental phage λNM567 itself will grow as a small plaque on a proN host as well as all recombinants carrying chiA as long as they arise from crossovers before the nin-deletion. We therefore decided to introduce the chiA mutation into a phage carrying a lacZ fragment of E. coli inserted between sri λ1 and sri λ3 with the following cross:

\[ \lambda \text{am} \times \lambda \text{L334} \]

Large blue clear plaques were selected on proN sup (ML10) on Xg-plates (M -12), purified and checked.

The resulting phage was then crossed with \( \lambda^{50} \text{im}434 \text{al} \text{ari} \lambda^{40} \text{Pan} 80 \text{ari} \lambda^{50} \) (λDB32= λNL17) to replace the right arm for one carrying the Pan mutation.

To avoid loss of the sri λ^{40} and sri λ^{50} mutations by multiple crossovers, the Pan phage used carried these same mutations in the right arm as well as an \( \lambda^{30} \) substitution to protect the left arm from unwanted genetic exchanges:
cross 2: $\lambda$WL1 $\times \lambda$DB82

Selection was on a $\lambda^{8\text{grop}}$ tonA host (WL9) and plaques were screened for the right immunity. This recombinant, $\lambda$WL23, was then crossed with $\lambda$NM567 to give

$\lambda$WL23, $\equiv \lambda$NM567chiA:

cross 3: $\lambda$NM567 $\times \lambda$WL23

Selection was on $\text{pro}^\text{rll sup}^0$ (WL10) for large white plaques on Xg-plates.

b) Introduction of shn$\lambda^6^0$ into $\lambda$WL23.

As a source of the shn$\lambda^6^0$ mutation $\lambda$WL37 was used. The preparation of this phase is described in $\lambda$-13.

An SamT mutation was first introduced into the right arm of $\lambda$WL23 with the following cross:
cross 4: \( \lambda \text{WL28} \times \lambda \text{DB83} \)

\[ h^{80} \text{bio1 cr} \text{srI4}^{0} \text{(nin5)}^{+} \text{srI} \lambda^{5^{0}} \text{Sam7} \ (\lambda \text{DB83}=\lambda \text{WL60}) \times \lambda \text{WL28}. \] The \( h^{80} \) left arm and bio substitution of \( \lambda \text{DB83} \) will protect \( \lambda \text{WL28} \) against undesired exchanges. The cross mixture was enriched for \( S^{-} \) phages on a \( \text{sup}^{0} \) tonA host (WL29) \((M-13)\) and plated on \( \text{sulIII tonA} \) (WL25). Phages were checked for immunity and for \( \text{Sam7} \) by a complementation test \((M-19)\).

This \( \text{Sam7} \) derivative of \( \lambda \text{WL28}, \lambda \text{WL40} \), was then crossed with \( \lambda \text{WL37}, \lambda \text{lacZ/ trpA-E} \) (red \( \text{sam cIII}^{+} \)imm21clts srI \( \lambda^{4^{0}} \text{(nin5)}^{+} \)shn \( \lambda^{6^{0}} \text{srI} \lambda^{5^{0}} \)).

\[ \lambda \text{WL28} \times \lambda \text{WL40} \]

Large white plaques were selected on a \( \text{groN sup}^{0} \) host (WL10) on Xg-plates and recombinants checked for immunity to produce \( \lambda \text{WL41}, h^{\lambda} \text{chiA31} \) (srA+)srI4- imm213hrI cr14^{0} (nin5)^{+} shn \( \lambda^{6^{0}} \text{srI} \lambda^{5^{0}} \) (fig. 1-2, p.1-2).

Of three isolates of this cross, \( \lambda \text{WL41 a,b and c} \), one lacked the \( \text{chi-site} \) (c) through a second recombination event to the left of lac (at about 39%) but before the \( \text{chi-site} \) (at about 36%). DNA preparations were made of all three isolates and the location of the restriction sites for \( \text{EcoRI} \) and \( \text{HindIII} \) confirmed
by rel electrophoresis (plate 1).

The theoretical fragment sizes of the left, central and right fragments made with EcoRI are 21.0 kb, 10.5 kb and 7.9 kb respectively, while those for HindIII are 22.9 kb, 9 kb and 8.4 kb.

Heteroduplex mapping showed later that both λL41a and b carry a duplication in the left arm, which is missing from λL41c (see chapter 2).
Fig. 1.2: the EcoRI-HindIII replacement vector \( \lambda WL 41 \)

a) schematic representation of the genome

b) diagram showing the location of the targets for EcoRI and HindIII.
**Legend to Plate I**

**Restriction analysis of the DNA of three isolates of λ WA1**

<table>
<thead>
<tr>
<th>Track</th>
<th>DNA</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>λ WA1a</td>
<td>EcoRI</td>
</tr>
<tr>
<td>2</td>
<td>λ WA1a</td>
<td>HindIII</td>
</tr>
<tr>
<td>3</td>
<td>λ WA1b</td>
<td>EcoRI</td>
</tr>
<tr>
<td>4</td>
<td>λ WA1b</td>
<td>HindIII</td>
</tr>
<tr>
<td>5</td>
<td>λ WA1c</td>
<td>EcoRI</td>
</tr>
<tr>
<td>6</td>
<td>λ WA1c</td>
<td>HindIII</td>
</tr>
<tr>
<td>7</td>
<td>λ⁺</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

The sizes of the EcoRI-generated λ⁺ fragments are shown at the right margin.
PLATE I
Chapter 2

Properties of \( \lambda \)VL41

To establish the characteristics of the \( \lambda \)VL41 vector it was used to clone DNA fragments from *Pseudomonas aeruginosa*.

In shotgun experiments where the restricted DNA of vector and donor is simply mixed for ligation, the central fragment of the phage carrying the red and gam genes is still present. Phages can therefore be reconstituted carrying the central fragment in the opposite orientation. Since such an inversion inverts the whole \( N \)-operon, we do not expect this to change the phenotype of such a phage. This was checked first by isolation of phages with an inverted central fragment.

a) \( \lambda \)VL41 derivatives with a central fragment inverted with EcoRI and HindIII.

One \( \mu \)g of DNA of \( \lambda \)VL41a was restricted with EcoRI or HindIII, religated, packaged in vitro and plated on C600 (WL1). Plaques were picked and the phages tested on recA (WL30) and C600(P2) (WL60): all were Spi\(^*\).

Gel analysis of the DNA showed two types of phages as judged from digests with BamHI, which cuts asymmetrically in the central fragment. Phages with both the normal and the inverted orientation of the central fragment were found. The properties of two such phages, \( \lambda \)L376, carrying an inverted central fragment flanked by the EcoRI sites and \( \lambda \)L378, carrying a HindIII-generated inverted fragment, are shown in table 2-I. This leads to the conclusion, that inversion of the central fragment of the phage with EcoRI or HindIII does not lead to a change in the vector phenotype and our selection on a (P2)-lysogen for recombinants is therefore a valid one.
Table 2-1: Phenotype of derivatives and recombinants of vector λWL41a

<table>
<thead>
<tr>
<th>phage</th>
<th>central fragment</th>
<th>Rec⁺</th>
<th>Rec⁺(P2)</th>
<th>recA</th>
<th>lig ts</th>
</tr>
</thead>
<tbody>
<tr>
<td>λWL41a</td>
<td>(srl λ2-srI imm34) RN</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>λL376</td>
<td>(srl λ2-srI imm34) i</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>λL373</td>
<td>(ssh λ3-shn imm34) i</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>λL371</td>
<td>(15 kb: EcoRI)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λL372</td>
<td>(4 kb: EcoRI)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λL374</td>
<td>(4.5 kb: HindIII)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λL375</td>
<td>(15 kb: HindIII)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Rec⁺: C600(WL1); Rec⁺(P2): C600(P2)(WL60); recA: C600(P2)(WL60); lig ts: K398(WL35).

n indicates the normal orientation; i indicates the inverted orientation.

b) Cloning DNA from Pseudomonas aeruginosa into λWL41a

Partial digests of Pseudomonas DNA were ligated onto λWL41a DNA and after transfection in a PFK mk⁺ host (WJP65) Spi⁻ recombinants were isolated on C600(P2) (WL60). All these recombinant phages showed the full Spi⁻ phenotype, failing on recA and lig ts hosts (WL30 and WL35). The properties of 4 such recombinants are listed in table 2-1.

c) The cloning capacity of λWL41a

Based on a vector length of 320 kb, λ⁺ (41 kb) and a minimum required length of the DNA to be packaged of 73% λ⁺ (39 kb) the DNA to be cloned can be theoretically only 2 kb smaller than the central fragment it replaces. The central fragments generated with EcoRI and HindIII are about 10.5 and 9 kb respectively, allowing a minimum insert size of approximately 3.5 and 7 kb. Gel analysis of several recombinants of the above cloning experiment showed inserts smaller than this.

As can be seen in table 2-1, λL372 carries a fragment of only 4 kb, while λL374 carries one of about 4.5 kb. Digests of the DNA of these two phages with HindIII BamHI and KpnI and combinations thereof, indicated extra DNA in the left arm of
both phages of about 10-13\* length (5-6 kb). In the case of \(\lambda L374\) it was concluded that this DNA was located somewhere between the first \(\text{BamHI}\) site at 11.4% on the \(\lambda^+\) map and the first \(\text{KpnI}\) site at 36% on the map. To establish whether this extra DNA is of foreign (e.g. \textit{E. coli}) origin or a tandem duplication (such as described by Bellett et al, 1971), DNA heteroduplexes were made and analysed in the electron microscope.

d) Heteroduplex analysis of \(\lambda L372\) and \(\lambda L374\).

It was decided to analyse heteroduplexes of \(\lambda WL41a \times \lambda L372\) and \(\lambda WL41a \times \lambda L374\). If the extra DNA is 'foreign', it will show as a single stranded loop at a fixed distance away from the insert (the \textit{Pseudomonas} DNA will loop out against the nonhomologous central fragment of \(\lambda WL41a\)). If on the other hand the extra DNA has arisen by tandem duplication of the adjacent region the loop will be at a number of different positions with respect to the insert. Furthermore, the loop can be of variable size in a \textit{Rec}+ host, depending on the number of duplications present (fig. 2-1 and 2-2).

![Diagram showing variable loop position in heteroduplexes of duplication mutants](image)

Diagram showing that in DNA heteroduplexes of the type \(\text{tde}(33)/\text{add}X(1)\), the extra DNA in \(\text{add}X(1)\) can be looped out to form a bush anywhere in the region that is duplicated. The two conditions for the formation of such a variable bush are: (1) the duplication in \(\text{add}X(1)\) must be a tandem duplication, and (2) \(\text{tde}33\) must contain one copy of the genes that are duplicated in \(\text{add}X(1)\).

Fig.2.1: Variable loop position in heteroduplexes of duplication mutants (see text) (from Bellett et al, 1971)
Diagram showing the formation of secondary duplication phages by recombination of a primary duplication phage (addX₀) with itself, in a rec⁺ host. Exactly one copy of the duplication can be added or lost by recombination between matching sequences anywhere in the region of the duplication, provided that the primary duplication phage carries a tandem duplication.

**Fig. 2.2:**

Formation of secondary duplications by recombination, resulting in loop of variable length in subsequent heteroduplex analysis (see text)

(from Bellett et al., 1971)

Twenty heteroduplexes of λL41a x λL372 and λL41a x λL274 were analysed and measured. Four such duplexes of λL41a x λL372 are shown in plate II. Each shows an additional piece of DNA, which loops out, in the left arm. The various parts are numbered schematically:

1
2
3
4
5
6

in which (1+2+3) = left arm (up to arIλ1)

with 1 = DNA left of the extra DNA
2 = nonhomologous loop of extra DNA
3 = area between (2) and arIλ1
4 = central fragment of λL41a (arIλ2-arIimm434)
5 = insert Pseudomonas DNA
6 = right arm (from arIimm434)

In a Rec⁺ host the length of (1+2+3) is not necessarily constant if the extra DNA is a tandem duplication (fig. 2). We cannot therefore use the length of (1+2+3) as a standard length in the molecule. The ends of the molecule could conceivably have lost stretches of DNA, excluding (6) as a standard. I therefore decided to use the length of the central fragment (4) as standard (one could alternatively use (5) but this fragment is much shorter, so the measuring error
Legend to plate II

Heteroduplex analysis of \( \lambda L372 \)

Four examples of the heteroduplex analysis of recombinant \( \lambda L372 \) versus the vector \( \lambda W14a \) to investigate the nature of the extra DNA in the left arm of \( \lambda L372 \) (see text) are shown.

The ratio's 2/4 and 3/4 were determined to establish whether distance (3) and the size of loop (2) are constant.

The variability in the size of the loop and its relative position indicate the presence of tandem duplications rather than insertion of foreign DNA.

The ratio's 3/4 and 2/4 are shown in the corners of the drawings of these 4 molecules on the page facing plate II.
will be larger). Determining the ratio's \(2/4\) and \(3/4\) will tell us whether
distance (3) and the size of the loop (2) are fixed or not, the latter
indicating a tandem duplication. The ratio's of the \(4\) molecules in plate II.
are shown in the corners of the drawings on the opposite page. From these data
and the data on the other 16 duplexes of \(\lambda\) mL41a x \(\lambda\) L372 and the 20 analysed
of \(\lambda\) mL41a x \(\lambda\) L374 it was concluded that the extra DNA had arisen by tandem
duplication.

e) **Heteroduplex analysis of \(\lambda\) mL41a, b and c**
The fact that several recombinants were found with inserts smaller than
expected made us wonder whether the duplication was already present in the
vector DNA. The DNA of \(\lambda\) mL41a, b and c was therefore analysed in hetero-
duplexes against \(\lambda^+\). Some molecules of the first two isolates showed a loop
in the same area as loop (2) in the previous duplexes, others didn't. \(\lambda\) mL41c
showed no duplication loops.

More detailed gel analysis of the left arms of these three phages showed that
the left arms of \(\lambda\) mL41a and b were longer than those of \(\lambda\) mL41c and \(\lambda^+\).
\(\lambda\) mL41c is the isolate which has lost the chi-site.

Duplications in the left arm have been described before (Franklin, 1967;
Bellett et al, 1971) and Blattner has used them to increase the size of some
of his vectors which would otherwise be too small to be packaged (Blattner et
al, 1977). In practice this duplication means a possible reduction in cloning
capacity (see further section f).

f) **Possible involvement of chi in duplication formation**
Phages with very small genomes (75-78 \(\lambda^+\) length) have been described, which
grow very poorly, but revert with high frequency from a small plaque phenotype
to a large one. Franklin (1967) described such a phage, \(\lambda\) del133, a deletion
derivative of \(\lambda\)' d30 hybrid (\(\lambda\) att \(\rightarrow\) \(\lambda\)del30), which is only 77% \(\lambda^+\) length. It
throws of large plaques at high frequency \((10^{-2})\). Phages in the large plaques
can be shown to have obtained duplications increasing their size and therefore their viability. Emons et al (1975) showed these duplications to be at random positions in the genome. They also compared \( \lambda_{tdel33} \) with another small genome phage, \( \lambda_{b221} \), which carries a deletion of 22%. This phage also reverts to a larger plaque former but at a 50-100 fold lower rate than \( \lambda_{tdel33} \) (1-2x \( 10^{-5} \)).

In a mixed infection of \( \lambda_{tdel33} \) and \( \lambda_{b221} \) the rate of 'reversion' of the latter is considerably increased (Emons & Thomas, 1975). Emons & Thomas therefore postulate a trans-active product, present in \( \lambda_{tdel33} \).

The fact that \( \lambda_{A\text{M41c}} \) has no duplication and no chi-site can easily be explained by the proximity of chi to the duplicated area. However, \( \lambda_{W141} \), though it has a small genome (32\% \( \lambda^+ \) length), is a vector well above the viable limit of 78\%.

No duplications have been found previously in vectors with small genomes (W.J. Brammar, pers. comm.), which all lacked chi-sites. It is of course possible that a small duplication, caused by e.g. a polymerase slip (Ghosal & Saedler, 1978), is more rapidly turned into a series of duplications by a Rec\(^+\) host as described in fig. 2, increasing the size of the phage more rapidly than when no chi-site is present. It did however make us wonder, whether there could be any involvement of chi in the formation of duplications. \( \lambda_{tdel33} \) has the right arm and central area of \( \phi80 \). \( \phi80 \), in contrast to \( \lambda \), carries many chi-sites.

Unger et al (1972) mention that the \( \lambda/\phi80 \) parent of \( \lambda_{tdel33} \), though red\(^-\), shows normal recombination in a Rec\(^+\) host, which indicates the presence of chi. \( \lambda_{b221} \), on the other hand has (at least normally) no chi-sites present. The postulated trans-activity of \( \lambda_{tdel33} \) on \( \lambda_{b221} \) could be explained by chi-stimulated exchanges between the homologous left arms of the two phages, producing more viable \( \lambda_{b221} \) phages in the progeny.

A possible way of testing this is by introducing a Dam mutation into two identical phages, with and without a chi-site, which are smaller than 32\% length. DNA of such a phage can be packaged in a sup\(^{0}\) host under high salt conditions, whereas larger DNA molecules cannot (Sternberg & Weisberg, 1977). Duplication formation could therefore be monitored by comparing the e.o.p. of the two...
lysates (of chi\(^+\) and chi\(^-\) phages) on a suppressing host) on sup\(^0\) and suII hosts under Rec\(^+\) and Rec\(^-\) conditions. If chi stimulates duplication formation, the titre of the chi-carrying phage on sup\(^0\) should drop faster than that on suII, compared to the controls.

Attempts to introduce Dam into two derivatives of \(\lambda\)ML47a, both lacking the duplication, one without (\(\lambda\)ML47.1) and one with a chi-site (\(\lambda\)ML57, see next chapter), failed both in vivo and in vitro. Results indicated that these genomes were too large to allow packaging in a sup\(^0\) host. ChiA might be to the left of KpnI site 1, which is at 36\% on the \(\lambda^+\) map. No more time could be spent on this interesting sideline. It is however clear that the chi-site might pose a danger to the cloning capacity of a vector with a small genome, propagated in a Rec\(^+\) host. If the duplicated area becomes longer than 7 kb (for HindIII) and 8.5 kb (for EcoRI), it would also destroy our selection for Spi\(^-\) phages, since it would then be possible to obtain viable phage by ligating left and right arms without the central fragments. This lead us to investigate the use of Rec\(^-\) hosts to avoid these problems (see chapter 4 & 5).
Chapter 3

A λ replacement vector for use with BamHI

The construction of \( \lambda \)NM570BV2 (Klein & Murray, 1980), an insertion vector for use with BamHI, opened the possibility of converting \( \lambda \)WL41 into a phage suitable as a large capacity replacement vector for use with this enzyme and the series of other enzymes producing the same sticky ends. Amongst the latter are two tetranucleotide-recognising enzymes MboI and Sau3A, which are potentially very useful for the establishment of genebanks.

We decided to introduce the sbhl \( \lambda^{1\circ} \) mutation from the left arm of \( \lambda \)NM570BV2 into \( \lambda \)WL41 isolate a (note: at that time the duplication in the \( \lambda \)WL41a DNA was not known).

a) Introduction of sbhl \( \lambda^{1\circ} \) into \( \lambda \)WL41a

After various unsuccessful attempts, derivatives of \( \lambda \)WL41a carrying double amber mutations in the left arm (so we could distinguish this arm from the left arm of \( \lambda \)NM570BV2) were isolated from the following cross:

\[
\begin{align*}
\text{cross } 6: \lambda \text{WL41} \times \lambda \text{WJB307} \\
\lambda \text{WJB307} \text{ is an } \text{Sam7} \text{ version of } \lambda \text{WJB308, which was used in the first cross in chapter 1. White plaques were selected on groE (WL48) on Xg-plates.}
\end{align*}
\]

Immunity and the presence of two amber mutations were confirmed and this phage, \( \lambda \)WL42, was crossed with \( \lambda \)L368, a clear mutant of \( \lambda \)NM570BV2, \( \lambda \text{sbhl} \lambda^{1\circ}(\text{sbhl} \lambda \text{2-3})^{\text{red3 imm21 ci}^{-}\text{(nin5)}^{\text{shn}} \lambda^{6\circ}}; \)
Large clear plaques were selected on a sup\textsuperscript{0} supernus host (WL61). Phages with the right immunity were isolated. When the DNA of three of these phages was checked, two of them (\(\lambda\)WL4\textsubscript{a} a & b) carried the right EcoRI and BamHI targets but had regained the shn\(\lambda 6^+\) site in the right arm, which must have arisen from a double crossover event in the previous cross as indicated by \(-\ldots-\) (the third isolate (c) showed several such multiple crossovers).

To obtain a vector for use with all three enzymes, the DNA of \(\lambda\)WL4\textsubscript{a} and \(\lambda\)WL4\textsubscript{a} was restricted with BamHI, mixed and religated:

\[
\begin{align*}
\text{(I)} & \quad \text{WL4} \\
\text{(II)} & \quad \text{WL41}
\end{align*}
\]

\[\text{cross } ?: \text{(in vitro)} \lambda\text{WL41} + \lambda\text{WL41}\]

To increase the number of phages carrying the shn\(\lambda 6^0\) mutation a three-fold excess of \(\lambda\)WL4\textsubscript{a} DNA was used for ligation. Use of BamHI will decrease the number of recombinants regaining the sbhi\(\lambda 1^+\) site present in \(\lambda\)WL4\textsubscript{a}.

All the outcoming phage, when checked on C600(P2) (WL60), were Spi\textsuperscript{+}.

DNA preparations were made from 12 isolates (\(\lambda\)WL47, isolates 1-12) and 5 of these, isolates 1, 6, 10, 11 and 12 showed the right combination of restriction targets. The restriction fragments generated from two of these
isolates, 1 and 6, are shown on plate III.

Track 1, 2 and 3 show EcoRI, HindIII and BamHI digestion of DNA of isolate 1; track 5, 6 and 7 those of DNA of isolate 6; track 4 is DNA digested with EcoRI.

b) Phages with a central fragment inverted with BamHI

XhoI digestion of the DNA of isolates 1, 6, 10, 11 and 12 of \( \lambda \) WLA7 showed that 1 and 11 carried the central fragment in the normal orientation, producing bands on the gel of approximately 29 and 11 kb. Isolates 6, 11 and 12, however, show bands of approximately 25 and 16 kb, indicating that the fragment was in the opposite orientation. Data are shown for isolates 1 and 6 in track 8 and 9 of plate III.

This was an important observation, since it demonstrated that the Spi\(^+\) phenotype is retained irrespective of the orientation of the central fragment. This means that our selection for Spi\(^-\) recombinants is not invalidated using BamHI, despite the fact that inversion of the central fragment separated the red and gam genes from their normal promoter \( p_L \). BamHI, in contrast to both EcoRI and HindIII, which cut outside the operon, cuts beyond \( N \), in the middle of the operon. The inverted red and gam genes of isolates 6, 11 and 12 must therefore be transcribed from a rightward promoter. This was further investigated and is described in section g of this chapter.

c) Cloning into vector \( \lambda \) WLAB.1 using BamHI

Random fragments of E.coli DNA (restricted with BamHI and (partial) MboI respectively) were cloned into \( \lambda \) WLAB.1 after cutting the vector DNA with BamHI and recombinants were isolated. These showed the same characteristics as the EcoRI- and HindIII-generated recombinants of \( \lambda \) WLAB.1a (see chapter 2), including the full Spi\(^-\) phenotype. Two such recombinants are listed in table 3-I.
Legend to Plate III

Restriction analysis of the DNA of two isolates of \( \lambda \text{WL47} \)

<table>
<thead>
<tr>
<th>Track</th>
<th>DNA</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( \lambda \text{WL47.1} )</td>
<td>EcoRI</td>
</tr>
<tr>
<td>2</td>
<td>( \lambda \text{WL47.1} )</td>
<td>HindIII</td>
</tr>
<tr>
<td>3</td>
<td>( \lambda \text{WL47.1} )</td>
<td>BamHI</td>
</tr>
<tr>
<td>4</td>
<td>( \lambda^+ )</td>
<td>EcoRI</td>
</tr>
<tr>
<td>5</td>
<td>( \lambda \text{WL47.6} )</td>
<td>EcoRI</td>
</tr>
<tr>
<td>6</td>
<td>( \lambda \text{WL47.6} )</td>
<td>HindIII</td>
</tr>
<tr>
<td>7</td>
<td>( \lambda \text{WL47.6} )</td>
<td>BamHI</td>
</tr>
<tr>
<td>8</td>
<td>( \lambda \text{WL47.1} )</td>
<td>XhoI</td>
</tr>
<tr>
<td>9</td>
<td>( \lambda \text{WL47.6} )</td>
<td>XhoI</td>
</tr>
</tbody>
</table>

The sizes of the EcoRI-generated \( \lambda^+ \) fragments are shown at the left margin.

A simplified map showing the position of the restriction targets for EcoRI, HindIII, BamHI and XhoI is shown underneath for \( \lambda^+ \), \( \lambda \text{WL47.1} \) and \( \lambda \text{WL47.6} \).

The map at the top shows some of the genetic markers of \( \lambda \), while the scales 0 to 100 and 0 to 50 represent the length of \( \lambda^+ \) DNA in \( \% \) \( \lambda \) units and in kb respectively.
PLATE III

A | J | att | red v | N cl | OP | Q SR
---|---|-----|-------|------|----|-------
0 | 0 | 10  | 20    | 30   | 40 | 50    | 60   | 70 | 80 | 90 | 100

**Diagram:**

- Lane 2: Marked with B, H, and X enzymes.
- Lane 4: Marked with E and B enzymes.
- Lane 6: Marked with E, B, and H enzymes.
- Lane 8: Marked with E and B enzymes.

**Annotations:**

Table 3-1: Phenotypes of the vector λ WL47 and its derivatives

<table>
<thead>
<tr>
<th>Phage</th>
<th>Central fragment</th>
<th>Rec⁺</th>
<th>Rec⁺(P2)</th>
<th>recA</th>
<th>lig ts</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ WL47.1</td>
<td>(sbhI + 3-4)ⁿ</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>λ WL47.6</td>
<td>(sbhI + 3-4)ⁱ</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>λ WL48</td>
<td>(10 kb: BamHI)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>λ WL50</td>
<td>(16 kb: MboI)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Rec⁺: C600(WL1); Rec⁺(P2): C600(P2)(WL60); recA: QR48(WL30); lig ts: N3098(WL35).

ⁿ indicates the normal orientation; i indicates the inverted orientation.

All recombinants plated on a groN host as large plaques, indicating the presence of a chi-site. λ WL47.1 and λ WL47.6 were spotplated as controls in this experiment. Both plated on groN, but λ WL47.6 as a much larger plaque than λ WL47.1. It became evident that λ WL47.1 had lost its chi-site, plating poorly on groN at single plaque level. Isolate 11 also showed a small plaque phenotype on this strain, while isolates 10 and 12 were large on groN. This made me realise that the large plaque phenotype of λ WL47.6 on a groN host was due not to the presence of a chi-site, which was lacking in isolate 1, but to N-independent red and gam expression in this phage. As discussed in section b, these genes are separated from their normal promotor pₗ and therefore do not use the N-gene product for expression.

The large plaque phenotype of the recombinants, which lack red and gam, is due to the presence of (a) chi-sequence(s) in the cloned E.coli fragment. Since chi-sites occur with a frequency of about one site per 5 kb in the E.coli chromosome (Malone et al, 1978), the chances of cloning a 10-15 kb fragment from E.coli with a chi-site are very high.

I decided to reintroduce chiA by selecting large plaque formers on groN of
\( \lambda WL47.1 \) and check them for the presence of chiA.

Apart from chiA these mutants could also be chiB (located at 65% on the \( A^+ \)-map), chiC in the cII-gene (at 80%) or chiD (at 90%) (Stahl et al, 1980).

d) Reintroduction of chiA into \( \lambda WL47.1 \)

\( \lambda WL47.1 \) was spotplated on a groN host. Large spontaneous mutant plaques were purified and retested on groN for a large plaque phenotype. Lysates were made of 25 isolates and tested for cII\(^-\) in a complementation test. The mutation generating a chiC-site will inactivate the cII-gene, leading to a clear phenotype. None of the checked isolates were cII\(^-\), so none of them were chiC.

The following cross was used to distinguish between chiA or chiB on the one hand and chiD on the other:

\[
\begin{array}{c}
\text{W3m} & \text{E3m} & \text{Lc} & \text{Z} & \text{X} \\
\text{WJ3B308} & \text{(cI}^4\text{min}^4) & \text{cII}^6 & \text{cII}^6 & \text{LRB308} \\
\end{array}
\]

\[
\begin{array}{c}
\text{1} & \text{2} & \text{3} & \text{4} \\
\text{(cI1X1-2)\( ^6 \)} & \text{(cI1X1-2)\( ^6 \)} & \text{cII}^6 & \text{cII}^6 \\
\end{array}
\]

\[
\begin{array}{c}
\text{X_A} & \text{X_B} & \text{X_C} & \text{X_D} \\
\text{XWJ47chi} & \text{XWJ47chi} & \text{XWJ47chi} & \text{XWJ47chi} \\
\end{array}
\]

Cross 9: Isolates \( \lambda WL47chi \times \lambda WJ303 \), to locate chi.

12 Isolates were crossed with \( \lambda WJ308 \) (WL26) and plated on a \text{sup}^0(\text{\lambda imm434}) (WL83) host on Xg-plates. Increased recombination in the left part of the phage will indicate a chiA or chiB mutation, in the right part chiD. This would result in relatively more blue recombinants in these crosses through chiA- or chiB-promoted recombination (crossovers 1 and 2) compared to that by chiD (more white recombinants, crossovers 3 and 4). As controls \( \lambda WL41a \) and \( \lambda WL47.1 \) were crossed with the same phage. \( \lambda WL41a \) gave nearly twice as many blue recombinants as \( \lambda WL47.1 \) under similar conditions. The \( \lambda WL47chi \)
isolates could be divided into two groups, with a relatively high or low number of blue plaques. Some of the former were tested further.

The blue recombinants from the crosses of these isolates with \( \lambda \text{WJB308} \), could have obtained \( \text{chia} \) if the parent carried \( \text{chia} \), but never \( \text{chib} \), since they are Lac\(^+\) (the lacZ fragment covers the area in which \( \text{chib} \) would be located). Two blue recombinants out of 45 blue recombinants tested of one of the isolates, \( \lambda \text{WL47chib} \), spotted as large plaques on groN. It was therefore concluded that \( \lambda \text{WL47chib} \) carried \( \text{chib} \).

Final proof was obtained by checking recombinants of this vector for their characteristics. Attempts to check \( \lambda \text{WL47chib} \) and \( \lambda \text{WL47.1} \) carrying the known chi-less \( E.\text{coli} \) fragments supF and tna (Anilionis, 1977) for chi failed, due to the fact that such phages are barely viable and no lysates could be obtained.

Therefore I did a 'shotgun'-cloning experiment using BamHI-digested \( E.\text{coli} \) DNA into \( \lambda \text{WL47chib} \) and \( \lambda \text{WL47.1} \) (as control) vector DNA. Two strongly growing recombinants were selected on C600(P2) (WL60) and shown to give large plaques on groN, indicating the presence of at least one chi-site (e.g. on the insert). Recombinants of \( \lambda \text{WL47.1}/E.\text{coli} \) (\( \lambda \text{WL55} \)) and \( \lambda \text{WL47chib}/E.\text{coli} \) (\( \lambda \text{WL56} \)) were crossed with \( \lambda \text{DB113}, h^{30}\text{int29\ cl857(nins)} \):
phenotype on groN if $\lambda$ WL56 carries chiA, while they will be small if $\lambda$ WL47chi6 contained chiB. The control cross $\lambda$ WL55 x $\lambda$ DB113 should give recombinants which are small on groN. All recombinants from the cross $\lambda$ WL56 x $\lambda$ DB113 were large, while all those of $\lambda$ WL55 x $\lambda$ DB113 were small, indicating that $\lambda$ WL47chi6 carries chiA. This phage was renumbered $\lambda$ WL57.

e) The left arm of $\lambda$ WL57

An important question was whether $\lambda$ WL57 carried the same duplication as $\lambda$ WL41a, or whether the parent $\lambda$ WL41.1 had lost it together with the chi-site.

Gel analysis showed the left arm of $\lambda$ WL57 to move parallel to that of $\lambda^+$ and slightly ahead of $\lambda$ WL41a.

The 16 kb insert in $\lambda$ WL50 (table 3-I) showed that the duplication, if present, was smaller than the one in $\lambda$ L372 or $\lambda$ L374. Heteroduplex analysis of $\lambda$ WL57 against $\lambda^+$ showed no duplication loop in the left arm.

f) The cloning capacity of $\lambda$ WL57

Since there is no duplication in the left arm of $\lambda$ WL57, the capacity of the $\lambda$ WL57 vector is therefore as can be theoretically calculated, based on a minimum required length of 78% $\lambda^+$ length and a maximum of 109% (Bellet et al., 1971; Weil et al., 1972). The cloning capacity of this multipurpose vector is shown in table 3-II (page 3-8).

g) Red and gam expression in $\lambda$ WL47.6

Phages carrying a central fragment inverted with BamHI will still express the N-gene from the normal promotor $p_L$, but the rest of the N-operon is now apparently transcribed from a rightward promoter. These phages are Spi+, indicating expression of red and gam.
Table 3-II: The cloning capacity of $\lambda$WL57

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cloning capacity (kb)</th>
<th>Characteristics of recombinants</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI$^1$</td>
<td>8.6 - 24.0</td>
<td>(att int red gam N cl)$^\wedge$</td>
</tr>
<tr>
<td>HindIII</td>
<td>7.1 - 21.6</td>
<td>(att int red gam N cl)$^\wedge$</td>
</tr>
<tr>
<td>BamHI</td>
<td>4.7 - 19.6</td>
<td>(int red gam)$^\wedge$</td>
</tr>
<tr>
<td>BglII$^2$</td>
<td>4.7 - 19.6</td>
<td>(int red gam)$^\wedge$</td>
</tr>
<tr>
<td>Sau3A/MboI$^2$</td>
<td>4.7 - 19.6</td>
<td>(int red gam)$^\wedge$</td>
</tr>
<tr>
<td>XhoI$^3$</td>
<td>0 - 13.3</td>
<td>cIII$^-$</td>
</tr>
<tr>
<td>SalI$^3$</td>
<td>0 - 13.8</td>
<td>(red gam)$^\wedge$</td>
</tr>
</tbody>
</table>

(1) The donor DNA can be cut with EcoRI$^+$
(2) In each case the vector DNA must be cut with BamHI
(3) The vector DNA can be cut with XhoI or SalI
(4) The target for XhoI lies in the cIII gene. There will be no detectable change in the phenotype of such recombinants since the vector is cI$^-$.  
(5) The Spi$^-$ selection resulting from the replacement of the red and gam genes cannot be used to select recombinants in this case, since phages of this phenotype arise by simple removal of the small fragment flanked by the two SalI sites of the vector phage (at 67.7 and 68.6% on the $\lambda^+$ map).

Cloning capacities have been calculated assuming a genome size of 48 kb for $\lambda^+$ DNA (Szybalski & Szybalski, 1979), a minimum size for packaging of 78% (Bellet et al, 1971) and a maximum size limit of 109% (Weil et al, 1972). These limits may vary with the constitution of the phage DNA and the physiology of the host.
To test the possibility that red and gam are read from \( P_R' \), the major promoter for late transcription, the following experiment was carried out.

Expression of \( P_R' \) is dependent on the positive regulator for late transcription, the \( Q \)-gene, which is phage specific (e.g. the product of \((Q)\lambda\) only works on \((P_R')\lambda\)). Therefore we tested \( \text{gam} \) derivatives of \( \lambda \text{WL47.1} \) and \( \lambda \text{WL47.6} \) for complementation of a \( \text{Spi}^-30 \) phage in a \( \text{recA} \) host.

Consider the following four situations in a \( \text{recA sup}^0 \) host (by choosing the \( \text{Spi}^- \) phage with a different immunity, e.g. \( \text{imm}^\lambda \), we can distinguish the two phages):

<table>
<thead>
<tr>
<th>Phage a ( (\lambda \text{imm}^434) )</th>
<th>Phage b ( (\lambda \text{imm}^\lambda) )</th>
<th>Complementation</th>
<th>Outcoming Phage</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda \text{WL47.1} \text{am} \lambda )</td>
<td>( \lambda \text{Spi}^- (Q^+) \lambda )</td>
<td>( + (\text{red, gam, } Q) )</td>
<td>( \text{imm}^\lambda, \text{imm}^434 )</td>
</tr>
<tr>
<td>( \lambda \text{WL47.1} \text{am} \lambda )</td>
<td>( \lambda \text{Spi}^- (Q^+)_{80} \lambda )</td>
<td>( + (\text{red, gam}) )</td>
<td>( \text{imm}^\lambda )</td>
</tr>
<tr>
<td>( \lambda \text{WL47.6} \text{am} \lambda )</td>
<td>( \lambda \text{Spi}^- Q^+ \lambda )</td>
<td>( + (\text{red, gam, } Q) )</td>
<td>( \text{imm}^\lambda, \text{imm}^434 )</td>
</tr>
<tr>
<td>( \lambda \text{WL47.6} \text{am} \lambda )</td>
<td>( \lambda \text{Spi}^- (Q^+)_{80} \lambda )</td>
<td>( + (\text{red, gam}) )</td>
<td>( \text{imm}^\lambda )</td>
</tr>
</tbody>
</table>

In test 1 phage a will complement phage b by supplying \( \text{red} \) and \( \text{gam} \), read from \( P_L \) and phage b will supply \( Q \) to a since both have the late control region of \( \lambda \). So both phages will grow.

In test 2 phage a will supply \( \text{red} \) and \( \text{gam} \) to phage b, but the \( Q_{60} \) protein supplied by phage b cannot work on phage a, so only phage b will grow.

In test 3 we have essentially a control like test 1. Both phages will grow, since \( Q \) is supplied in trans to phage a by phage b, \( \text{red} \) and \( \text{gam} \) by phage a to phage b.

In test 4 the \( Q_{60} \) product can not work on \( P_R' \) (of \( \lambda \) origin) of phage a. Therefore, if phage a is to supply \( \text{red} \) and \( \text{gam} \) in trans to phage b, which can not occur from \( P_L \) in this case, it will have to be from a \( Q^- \) independent promoter for rightward transcription. So, if phage b grows in this test, expression of \( \text{red} \) and \( \text{gam} \) is \( Q^- \)-independent.
and therefore not \( p_R \). If expression is \( Q \)-dependent, neither of the two phages will grow.

This was tested using a complementation test as described in M-19.

In such a complementation streak-test complementation in test 4 will indicate \( Q \)-independent expression of red and gam, no complementation \( Q \)-dependent promoter for the red and gam genes. The results are shown in table 3-III; both positive and negative controls were included and gave results as expected.

**Table 3-III**: Red and gam expression in \( \lambda \) WL47.6

<table>
<thead>
<tr>
<th>Stock nr.</th>
<th>Relevant characteristics</th>
<th>complementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda ) L396 x ( \lambda ) L398</td>
<td>( \lambda ) WL47.1 (Gam) ( \times ) Spi( ^- ) (QSR( ^+ ))( 80 )</td>
<td>+</td>
</tr>
<tr>
<td>( \lambda ) L396 x ( \lambda ) L102</td>
<td>( \lambda ) WL47.1 (Gam) ( \times ) Spi( ^- ) (Gam) ( Q )</td>
<td>-</td>
</tr>
<tr>
<td>( \lambda ) L395 x ( \lambda ) L398</td>
<td>( \lambda ) WL47.6 (Gam) ( \times ) Spi( ^- ) (Q( ^+ ))( Q )</td>
<td>+</td>
</tr>
<tr>
<td>( \lambda ) L395 x ( \lambda ) L102</td>
<td>( \lambda ) WL47.6 (Gam) ( \times ) Spi( ^- ) (Gam) ( Q )</td>
<td>-</td>
</tr>
<tr>
<td>( \lambda ) L395 x ( \lambda ) WL48</td>
<td>( \lambda ) WL47.6 (Gam) ( \times ) Spi( ^- ) (Q( ^+ ))( Q )</td>
<td>+</td>
</tr>
<tr>
<td>( \lambda ) WL48 x ( \lambda ) L398</td>
<td>( \lambda ) Spi( ^- ) (Q( ^+ ))( Q ) ( \times ) Spi( ^- ) (QSR( ^+ ))( 80 )</td>
<td>-</td>
</tr>
</tbody>
</table>

* \( \lambda \) L102 and \( \lambda \) L398 carry trpBG2, deleting red and gam; \( \lambda \) WL48 is a Spi\( ^- \) recombinant of \( \lambda \) WL47.1 (see table 3-I)

**a recA sup\( ^0 \) host (WL42) was used in this test

Conclusion:

\( \lambda \) L395 complements \( \lambda \) L398, therefore red and gam are expressed from a \( Q \)-independent promoter.
Recently Kravchenko et al (1979) produced evidence for a rightward promoter near att. This could be the promoter we are looking for, since inversion with BamHI of the central fragment leaves att at its normal position, just to the left of the inversion.

If we look at transcription patterns of the three phages with inversions generated with EcoRI, HindIII and BamHI (fig. 3-1), we see that inversion with EcoRI (iR) or HindIII (iH) produces phages with all major transcription in the rightward direction:

Fig. 3.1: expression of the red and gam genes in λDL47 and derivatives with inverted central fragment.
In the case of BamHI-generated inversions (iB), most transcription is from the rightward strand, with the exception of the expression of N from P_L. Since there is no transcription stop for P_L in the leftward direction, as long as N is present, we have a case of convergent transcription. Ward and Murray (1979) studied the problem of convergent transcription in λ. They compared transcription from P_L with and without a trp promoter reading in the opposite direction. Expression of trp is completely blocked while P_L is working. Transcription from P_L of the lacZ gene is delayed if P_trp is working. We could expect a rightward promoter from which red and gam are expressed to be blocked by transcription from P_L, but since the phage is cro⁺, P_L is switched off and red and gam could be expressed, though with delay. This does not seem to seriously affect growth of the phage, though λWL47.6 usually gives two types of plaques, one of normal size, like λWL47.1, and a very small one. The latter might be plaques which have suffered from the delay in red and gam expression.

h) λWL57 as an expression vector

1) EcoRI-generated recombinants

Using λWL57 as a vector for EcoRI-generated fragments, we remove the strong leftward phage promoter P_L. Transcription on the cloned fragment is therefore dependent on promoters located on the fragment, when inserted in the leftward orientation.

The (srl λ1-2)⁻ removes a transcription terminator in the b2 region for rightward transcription from P_R'. The second known transcription terminator for rightward transcription lies to the right of this deletion on the replaceable central fragment. This should allow transcription from P_R' to proceed into the cloned region.

2) HindIII-generated recombinants

As is the case for EcoRI we remove P_L, but in this case the cloned DNA
lies beyond the stop signal for rightward transcription from $P_R$' between sri $\lambda 2$ and shn $\lambda 3$, so we cannot express the cloned DNA from a phage promoter in either orientation.

3) Recombinants of $\lambda W L57$ using the sbhl $\lambda 3$ and sbhl $\lambda 4$ sites for cloning.

In this case $P_L$ and also $N$ are intact, so we can use this powerful promoter for leftward transcription, allowing transcription to proceed through the normal stop signals by the action of the $N$ gene product.

On the basis of our data on red and gam expression in $\lambda W L47.6$ we may assume that in the rightward orientation the cloned DNA can be expressed from the same promoter as the one from which red and gam are expressed in that case, presumably $P_{att}$, unless this promoter is a new promoter generated by sequences to the left of sbhl $\lambda 3$ and to the left of sbhl $\lambda 4$.

These results have been published in Gene 10 (1978) 249-259.
E. coli strains for the propagation of vectors and recombinants

a) Rec\(^+\) hosts

As bacterial hosts for vectors and recombinants we used originally conventional Rec\(^+\) hosts, such as C600, CP47(rk\(^{-}\)mk\(^{+}\)) or 303-derivatives (rk\(^{-}\)mk\(^{-}\) or rk\(^{+}\)mk\(^{+}\)). Recombinants can only grow on rk\(^+\) strains if the DNA is K-modified. Recombinant phage from an in vitro packaging experiment can therefore not be plated directly on the selective host C600(P2), but has to be modified first against K-restriction.

I therefore decided to make a (P2) lysogen of an 3C3-derivative which was rk\(^{-}\)mk\(^{+}\)tonA (WL97). This (P2) lysogen of WL97, WL95 plates Spi\(^-\) phage with normal efficiency but plaques on this host are much smaller than on C600(P2). After initial selection of recombinants on WL95 plaques were therefore always purified and grown on a different host (e.g. C600). Several attempts to obtain a 5K(P2) were unsuccessful (5K is an rk\(^{-}\)mk\(^{+}\) derivative of C600), though it should be possible to isolate such lysogens.

In the light of the sound duplications (as discussed in chapter 2) we decided to test various recAB and recBC hosts to minimize genetic exchanges.

b) Rec\(^-\) hosts

As pointed out in the introduction (p. I-13) Spi\(^-\) phage can not be grown on a recA host unless the strain is defective in recBC as well.

I tested various recBC hosts and one recAB host, which were already in existence.

The recAB strain, JC5495(WL57) is rk\(^{+}\)rs\(^{+}\)recAB and proved to be an excellent host strain. It grows very well and plates both vectors and recombinants with an e.o.p. of 1 as compared to C600 (see table 4-1). The fact however that a
strain defective in the major recombination system is such a healthy strain raised the suspicion that it carries a $\text{shca}$-mutation, reverting it to a recombination-proficient phenotype. From a cross $\lambda_{\text{bio22241057}} \times \lambda_{\text{bio101543}}^{\text{shca}}$ in WL57 (and a control strain) recombinants were obtained on $\text{prof tonA}$ at about normal frequencies. Thus our isolate of JC5495 appears to be recombination proficient in a $\lambda$-cross.

Three recBC strains were also checked as hosts. They were recBC derivatives of 803 (WL66, $\text{rk}^{-}\text{rk}^{-}$), 600 (WL73, $\text{rk}^{+}\text{rk}^{+}$) and 801 (WL74, $\text{rk}^{+}\text{rk}^{+}$). Vectors and recombinants grew less well on WL66, giving a smaller plaque and a slightly lower e.o.p. than on 8000. This could be related to the fact that this strain grows less fast than 8000 (the recombinants will be unable to use the chi-sequence in this strain).

WL73 and WL74 were worse hosts than WL66.

Anne Smith in this laboratory constructed recBC and recAB strains in an E. coli C background. RecBC was introduced via $\Phi_1$-transduction, recAB using an $\Phi^{fr}$ recAB.

These newly constructed strains proved worse than the previously tried recAB and recBC hosts.

The recBC strains WL67 and WL68 gave very small plaques and a low e.o.p., not only of the recombinants, but also of the vectors themselves.

The recAB hosts, WL69-72, grew very poorly giving a low phage yield. They reverted to a larger colony type at high frequency, probably by acquiring a $\text{shca}$-mutation. Use of freshly grown cells, rather than plating cells, gave a better result (see table 4-1).

Attempts at plate-lysates and liquid lysates of recombinant phages using WL67 and WL71 gave very low titres ($10^2 - 10^3 \text{d/ml}$) and so these strains are far from ideal hosts, though DNA preparations have been made using WL67 (A. Jeffreys, pers. comm.).

All these problems with Rec$^{-}$ hosts led to the idea of reintroduction of the
gam-gene into the vector, which can then be used in a suitable recA host. There are no convenient restriction targets immediately flanking the gam-gene, which would enable us to cut out the gene and transfer it to a convenient position in the right arm under \( p_{\lambda} \) control. It was therefore decided to try to introduce the gam-gene into a recA host on a plasmid or as a defective lysoren (see chapter 5).
Table 4.1: Plating efficiency and plaque size of vectors and recombinants on Rec^- hosts

<table>
<thead>
<tr>
<th>Host strain</th>
<th>Background characteristics</th>
<th>Relevant vectors</th>
<th>e.o.n. 1)</th>
<th>Plaque size 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WL1</td>
<td>C600</td>
<td>Fk^+ Fk^+ Rec+</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>WL57</td>
<td>JC5495</td>
<td>Fk^+ Fk^- RecAB</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>WL/c</td>
<td>903</td>
<td>Fk^- Fk^- RecBC</td>
<td>0.9-1.0</td>
<td>m</td>
</tr>
<tr>
<td>WL/c (1)</td>
<td>E. coli C</td>
<td>Fk^- Fk^- RecBC</td>
<td>0.5</td>
<td>s</td>
</tr>
<tr>
<td>WL/c (2)</td>
<td>E. coli C</td>
<td>Fk^- Fk^- RecAB</td>
<td>0.3</td>
<td>s,r</td>
</tr>
<tr>
<td>WL70 (3)</td>
<td>E. coli C</td>
<td>Fk^- Fk^- RecAB</td>
<td>0.6</td>
<td>s</td>
</tr>
<tr>
<td>WL70 (4)</td>
<td>E. coli C</td>
<td>Fk^- Fk^- RecBC</td>
<td>0.7</td>
<td>m</td>
</tr>
<tr>
<td>WL70 (5)</td>
<td>Brh1</td>
<td>Fk^- Fk^- RecBC</td>
<td>0.7</td>
<td>r</td>
</tr>
</tbody>
</table>

1) e.o.n.'s are compared to that of C600.
2) l = large; m = medium; s = small; r = pinpoint.
3) Vectors: λH41a, λH41c, λH47.1, λH47.c and λL37.c.
4) Rec. = recombinants; recombinants λL371, λL375, λH48 and λW50 (cf. chapters 2 & 3).
5) Fresh cells.

Note: Plating cells were used in all cases but one (WL70); plating cells were not more than two weeks old in the case of C600 and WL57, and less than one week old for the other strains. WL/c-71 give a better result when fresh cells are used.
Chapter 5

A recA host for the propagation of Spi− phages

The rk−mk− and rk−mk+ hosts, deficient in recBC or recAB, which were constructed and tested in chapter 4, have proven not to be very satisfactory for the growth of either vectors or recombinants. Due to the recBC mutation the chi-site in the recombinant phages cannot be used to stimulate recombination of the phage DNA to form packageable dimers, but the vectors themselves do not grow well, despite being red+gam+, on these hosts.

The absence of recA produces hosts which are very feeble and mutate to a recombination-proficient phenotype, probably via a sbcA mutation.

It was therefore decided to investigate the possibility of providing the λgam gene product in trans to recombinants in a recA-deficient host to allow rolling circle formation. This approach would allow us to keep our positive selection for Spi− recombinants on a Rec+(P2) lysogen, allowing the recombinants to use the chi-sequence, while subsequent growth on a recA host, carrying the λgam-gene, should minimize genetic rearrangements, such as duplications, whether or not they are related to the presence of the chi-site.

A recA host was therefore constructed carrying the λgam gene under the control of the λcl gene. By using a temperature-sensitive mutation in the repressor gene expression of gam can be regulated. This might be a necessary precaution: continuous expression of gam could be disadvantageous to the cell.

There are two possible ways of supplying gam in trans to Spi− phages in a recA host:

a) A defective lysogen

To allow cells to survive induction of a lysogen, the phage has to be defective for excision functions int and xis as well as the replication genes C and/or P. Strains have been constructed which provide trp-products from a
lysogen in a trp host using $\lambda h^{80} \text{BG2 ciB57}$ (Davison et al., 1974). In $\lambda h^{80} \text{BG2 ciB57}$ the trp genes are expressed from $\text{P}_i$; the $\text{N}$ gene is present, all DNA to the left of $\text{N}$, including $\text{red}$ and $\text{gam}$, is missing.

Induction and selection for survivors (at 42°C) which grow on medium lacking trp, produces cells carrying the prophage with a deletion covering cro, O and P. Spiegelmann (1971) noticed that induced $\lambda \text{cIts N}'$ lysogens will survive only if, in addition to being O' or P', the lysogens are also deleted for material to the left of $\text{N}$. Further studies by Greer (1975) narrowed the location of this function down using deletions covering the area of the gam gene.

She isolated point mutants which gave increased survival of lysogens and which did not affect gam functions as judged from ability of such mutants to grow normally on polA or lig ts hosts. Neither known gam' or cIII' mutants gave such increased survival and she proposed a new gene, the kil gene, the product of which she suggested might be a nuclease. The physical map in the att-N region appears to be fully occupied (Szybalski & Szybalski, 1979), so it is likely that kil overlaps gam. We could try to make a defective lysogen using $h^{80} \text{trp46(gam')ciB57 cro'}$ and select 42°C survivors, as was done for $\lambda \text{BG2}$.

Such survivors will however have lost in addition to O or P also kil and therefore possibly gam.

I made lysogens of such a phage ($\lambda \text{WL90}$) in a Rec' host, AB1157, lacking the cryptic prophage (Kaiser & Murray, 1980; Anilionis et al., 1980), genes of which are involved in the recE pathway. Subsequent transfer of recA into this host via conjugation produced only two isolates which were recA' amongst 30 conjugants checked, which however had both lost the trp-prophage. Though the phage's functions are supposedly repressed and therefore should not produce kil-functions, it is possible that low level expression of this gene causes damage which is easily repaired in a Rec' host or by the phage's red system, which in this case are both missing. Support for this assumption comes from experiments described later.

Therefore I decided to introduce kil335, a kil-ochre mutation, into the $\lambda \text{trp46}$
phage and confirmed increased survival of lysogens carrying such a phage ($\lambda$WL103) in comparison with the $\text{kil}^+$ version (data not shown).

The next step would be selection for survivors at 42°C and screening for those retaining immunity, followed by conjugation to transfer $\text{recA}$ into this strain. A quicker method to supply $\text{gam}$ in trans however might be the use of a plasmid carrying the $\text{gam}$ gene.

b) A plasmid carrying the $\lambda$gam gene

An alternative way to provide $\text{gam}$ in trans would be to transfer the $\text{gam}$ gene onto a plasmid. The EcoRI fragment (srI $\lambda$ 3-srI $\lambda$4) of $\lambda$cl857 DNA can be isolated (on a gel) and introduced into a plasmid vector such as pBR322. The latter provides no selection for recombinants using the EcoRI site but we can select for immunity against $\lambda$ of such a recombinant strain at 32°C. When using this (srI $\lambda$ 3-srI $\lambda$4) fragment we might consider the presence or absence of the $\text{cro}$ gene. The $\text{cro}$ gene product will switch off expression from $E_4$, the promoter for transcription of the $\text{N}$-operon, some time after induction at 37°C (in normal $\lambda$ infections after 10-15'). This will lead to relatively low levels of $\text{gam}$ product in the cell whereas high level continuous expression will be obtained when the fragment contains a deletion of the $\text{cro}$ gene. The continuous expression might be necessary to the phage, in which case a $\text{cro}^+$ plasmid will have to be used. The preparation of such a plasmid is relatively simple compared to the construction of the defective lysogen, since it does not involve screening of survivors (at 42°C) for immunity (at 32°C). DNA of pAT153, a derivative of pBR322, was available and so I introduced the fragment into this plasmid vector.

1) Plasmid vector pAT153 containing $\text{gam}$ cl857($\text{cro}$)$^+$

As the source of the DNA containing the $\text{gam}$ gene I used two fragments generated with EcoRI and BamHI from $\lambda$pen ($\lambda$L199) and pL-asmid (WL107).

The nature of pL-asmid is discussed in chapter 6. It carries the (sbhI $\lambda$4-
-srl^4) fragment of c1857 and has been deleted in vitro for the cro gene by removal of a BglII fragment. The fragment is cloned between the EcoRI and BamHI sites of pBR322. This 4.2 kb fragment was separated from the rest of the plasmid on a gel (since the plasmid (4.4kb) does not separate well from this fragment it was also digested with SalI). The adjacent 3.9 kb fragment (srl^3-sbhI') carrying the gam gene was recovered likewise from ^pen DNA. The two fragments were ligated onto pAT153, which was restricted with EcoRI to give the following potential plasmid (fig. 5-1).

![Diagram](image)

**Fig. 5.1**: A plasmid carrying the lam-gene under control of the c1 repressor.

The ligated DNA was transformed into the competent recA host HB101 (WL125) and into a Rec^+ control strain (WL96). Transformation followed by selection for immunity at 32^oC did not produce any recA colonies immune to lam though control L+amp plates showed the presence of transformed amp^R colonies. The Rec^+ control gave isolates immune to lam (WL136).

Attempts to transfer the plasmid pWL136 from the Rec^+ strain into WL125 produced amp^R transformants (WL146) but they were no longer immune to lam at
32°C.

So such a $\text{gam}^+\text{cl857(cro)}^+$ recombinant plasmid is apparently viable in a Rec$^+$ host, but lethal in a recA background, despite the fact that at 32°C the $\text{ci}$-repressor is active. Maybe the copy number is too high (or replication too fast) to allow repressor to block all transcription from $\text{pl}$. WL136 grows well at 32°C but gave a poor lawn when tested at 42°C. Derivatives were isolated which grew better at 42°C. Transfer of the plasmid from this strain into the recA strain WL125 gave similar results, producing no $\lambda$-immune amp$^R$ colonies.

The poor growth of WL136 at 37°C could be explained by continuous production of $\text{gam}$. This could be unfavourable to the cell. I therefore repeated the experiment using a cro$^+$ version of the plasmid.

2) A plasmid carrying $\text{gam}^+\text{cl857 cro}^+$

The 7 kb EcoRI fragment of WL120 containing $\text{gam}^+\text{cl857cro}^+$ was cloned into pAT153. The resulting plasmid was viable in Rec$^+$ but not in recA strains. WL165 is amp$^R$ Rec$^+$ and immune to $\lambda$ at 32°C. No such isolates could be recovered in a recA background.

WL165 grew poorly at 42°C like WL136, even though there is cro-repression in this case. Continuous expression of $\text{gam}$ is therefore not the cause of the feeble growth at 42°C of WL136.

A possible cause for the lethality of the plasmids pWL136 and pWL165 in a recA background and the poor growth at 42°C in a Rec$^+$ background is the presence of the kil-gene. If this is the explanation, we must then assume leaky expression at 32°C to produce enough kil-product to be lethal to a recA host but not to a Rec$^+$ strain, as has been discussed in section a for the defective lysogen. Damage by the kil-gene product could be repaired by the recA protein or possibly red function. If such repair functions are absent, the cell dies. It was therefore decided to repeat the experiment using a kil$^-$-derivative of $\lambda$. We used the same kil-ochre mutation kil335,
which leaves \textit{gam} intact.

3) A plasmid carrying the \textit{$\lambda$gam} gene in conjunction with a \textit{kil}$^-$ mutation

A 'shotgun' cloning experiment of $\lambda$\textit{kil335 ci857 (\textit{$\lambda$} WL100)} into pAT153 with \textit{EcoRI} was carried out. The ligated DNA was transformed into WL125 (\textit{recA}) and WL96 (a \textit{Rec}+ control) and produced, upon selection with phage, transformants which were immune to $\lambda$ at 32°C in both a \textit{recA} and a \textit{Rec}+ background. This suggests that leaky expression of \textit{kil} is the cause of lethality of pWL136 and pWL165 in a \textit{recA} background and supports the idea of the nuclease function of the \textit{kil} product.

WL214 (\textit{Rec}+) and WL215 (\textit{recA}) strains carrying this plasmid were tested for behaviour at 37°C and 42°C. Both grew poorly at 42°C and not very well at 37°C. The \textit{recA} host was more feeble than the \textit{Rec}+, but the same was the case for the parental strain WL125 as compared to the \textit{Rec}+ control (WL96).

Cells from fresh overnight cultures (grown at 32°C), were diluted 1:1 in fresh LB and grown 30-40' at 37°C. Reasonable lawns were obtained upon subsequent ON incubation at 37°C.

Both strains were immune to $\lambda$ at 32°C but also at 37°C. This was attributed to overproduction of the \textit{cro} gene product at 37°C. This was confirmed by testing for growth of $\lambda$\textit{vir} (Zissler, 1971a; Gottesmann et al, 1974). This phage is insensitive to repression by the \textit{cl}-product but responds to switch off by high levels of \textit{cro} product. $\lambda$\textit{vir} grows well at 32°C on WL214 and WL215. It does grow at 37°C but plaques are turbid and not properly developed. At 42°C there is virtually no phage growth, though this could be attributed to the poor condition of the cells at that temperature.

An ON culture of WL215 which was diluted 1:1 for 40' at 37°C allowed $\lambda$WL37 (Spi-) to grow. The phage was spotplated on WL215 and controls and produced plaques up to single plaque level (see table 5-I). When the same ON was diluted 1:20 and grown till $OD_{260} = 0.5$ before diluting 1:1 and then grown at 37°C for 40', it did however not plate $\lambda$WL37 and gave very poor lawns.
I decided to try a different recA host and see whether this would be healthier at 37°C. QR48, a recA derivative of QR47 (WL30) was chosen for this purpose. It was transformed with pWL215 to produce WL217.

This strain WL217 gave similar results to WL215, but the strain grew better at 37°C than WL215. No plaques of \( \lambda \) WL37 were obtained either by spot-plating or at a single plaque level when using a fresh culture, which was grown for 40' at 37°C after initial growth till early log. phase at 32°C. Plaques were found however using cells which were grown ON at 32°C and had not been grown at 37°C prior to infection with \( \lambda \) WL37.

Plaques were consistently larger on WL217 than on WL215, both for Spi\(^+\) and Spi\(^-\) phages. Plaques of \( \lambda \)WL48, a Spi\(^-\) recombinant of WL47.1, were very small on WL217 and barely visible on WL215. On a recA control strain no growth of \( \lambda \) WL37 or \( \lambda \) WL48 was observed (see table 5-I).

These results indicate that:

a) the cro gene product is responsible for the failure of \( \lambda \) WL37 and \( \lambda \) WL48 to grow on such a recA (p(gam)) strain under certain circumstances. If the cells are rapidly dividing at 32°C, 40' incubation at 37°C will have inactivated the cl repressor, leading to the production of N and gam, but also to that of cro. We have to assume from our data that the cro gene product has accumulated sufficiently in those 40', to act at \( E_L \) and prevent the expression of gam, that would be necessary for \( \lambda \) WL37 and \( \lambda \) WL48 to grow on a recA host. In log.phase cells we have to assume that all gam product has been inactivated by the recBC exonuclease after 40' at 37°C with further production of gam protein being repressed by cro. At the moment that \( \lambda \) WL37 would have to switch to rolling circle replication using gam, provided in trans by the plasmid, this step is therefore blocked by the action of the cro product. If, on the other hand we use an ON culture (grown at 32°C) the stationary cells are after 40' at 37°C still in a lag phase or just entering the log.phase. Cro product has not yet accumulated allowing production of gam, needed for growth of
\WL37. Once \WL37 has switched to the rolling circle mode, it is insensitive to \texttt{recBC} nuclease action. A high number of replicating \Spi phage genomes will titrate the \texttt{cro} product, postponing the moment that \texttt{pL} will be blocked completely by high levels of \texttt{cro}.

b) A factor, which is not \texttt{kil}, has some influence on cell growth at 37\textdegree{}C, leading to feeble growth at this temperature and low phage yield of \Spi phage. This factor might well be the strong \texttt{pL} promoter. There are indications that strong promoters on high copy-number plasmids are deleterious to the cell, draining the cell of precursor molecules and active RNA polymerase (West et al., 1980).

\texttt{pL}-asmid, designed as a high level expression vector using \texttt{pL}, has not fulfilled its promise, growing poorer at 37\textdegree{}C than at 32\textdegree{}C, leading to lower yields than expected of the products of cloned genes (e.g. \texttt{Th4-DNA ligase} (Alan Hall, pers. comm. J. Windass) and see also chapter 6).

These results lead to the conclusion that a \texttt{cro} version of the WL215 plasmid (\texttt{gam\^{}kil\^{}cl857}) is necessary for sufficient levels of \texttt{gam} protein, but that we might have to increase the viability of such a strain by reducing the level of transcription from the promoter from which \texttt{gam} is expressed, \texttt{pL}.

This could be done in several ways:

a) a \texttt{pL-sex} mutation. This promoter mutation leads to a ten-fold decrease in the rate of expression from \texttt{pL}.

b) introduction of a \texttt{N}-independent transcription terminator. Such a terminator on the plasmid would prevent transcription without reinitiation and so slow down transcription.

c) a \texttt{N} mutant. This would allow transcription from \texttt{pL}, but in the absence of \texttt{N}-protein this will terminate at \texttt{\texttt{I}^{}L1}. Leaky transcription through this stop signal might give sufficient levels of \texttt{gam} protein to allow growth of a \Spi phage (This situation can be mimicked using a \texttt{groM} host strain).
d) a low copy number plasmid. We could use a low copy number plasmid like pSC101 to give low level expression of \textit{gam} either from $\beta_L$ or from a plasmid promoter.

e) a different promoter. By using a naturally weak promoter we could try low level continuous expression of the \textit{gam} gene. The \textit{gam}-gene could be recovered from $\lambda$WL100 on a BclI-BamHI fragment and cloned into the BamHI site in the \textit{tet}-gene of pBR322 under control of the \textit{tet}-promoter.

The combination of a $\beta_L$-sex mutation in conjunction with a stop signal in a $\textit{cro}^-$ background seems a good choice for a lower level controllable expression of \textit{gam}, or $\textit{N}^\text{co}$ allowing transcription termination at a terminator on the plasmid (e.g. near the origin (Stüber & Bujard, 1981)) (both in conjunction with $\textit{kil}^-$).
Table 5-I: Properties of WL214, WL215 and WL217, carrying gam kil- cl857 cro+

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Phage</th>
<th>( \lambda \text{imm}^+ \text{Spi}^+ )</th>
<th>( \lambda \text{imm}^{434} \text{Spi}^+ )</th>
<th>( \lambda \text{imm}21 \text{Spi}^- )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WL96</td>
<td>Rec(^+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>WL125</td>
<td>recA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WL30</td>
<td>recA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>WL214</td>
<td>WL96 (p(gam))(^{***})</td>
<td>-</td>
<td>+</td>
<td>+(^{****})</td>
<td>+</td>
</tr>
<tr>
<td>WL215</td>
<td>WL125 (p(gam))(^{***})</td>
<td>-</td>
<td>+</td>
<td>+(^{****})</td>
<td>-</td>
</tr>
<tr>
<td>WL217</td>
<td>WL30 (pWL215)</td>
<td>-</td>
<td>+</td>
<td>+(^{****})</td>
<td>-</td>
</tr>
</tbody>
</table>

\* \( \lambda \text{imm}21 \) gave similar results

\** \( \lambda \text{imm}^{434} \) gave similar results (see text)

\*** these plasmids might be identical, both were recovered from the same ligation experiment.

\**** slightly reduced growth at 32°C and 37°C

\( \lambda \) Spi\(^+\) phages: \( \lambda \) WL73, \( \lambda \) WL57, \( \lambda \) WL24 and \( \lambda \) WL25

\( \lambda \) Spi\(^-\) phages: \( \lambda \) WL37 and \( \lambda \) WLA8.
Chapter 6

The cloning of the genes for restriction enzymes and their modification enzymes into λ

Using vector λ WL57 I decided to investigate the possibility of cloning the genes for some restriction enzymes into λ to study the regulation of expression of genes for these enzymes and their modification enzymes. A heat-inducible lysogen should be an enriched source for the enzyme, analogously to the successful production of the T4 DNA-ligase (Panasenko et al., 1977; Murray et al., 1979).

a) Attempts to clone the genes for HindIII, BamHI and Sall into λ

As a first choice the genes for HindIII, BamHI and Sall were chosen since the vector arms of λ WL57 have no targets for these enzymes, increasing the chance of obtaining the desired recombinants.

'Shotgun' cloning experiments were carried out with DNA from Hemophilus influenzae Rd, Bacillus amyloliquefaciens H and Streptomyces albus. DNAs were digested with EcoRI, HindIII, BamHI and MboI (partial). As vector I used a SamT-derivative of λ WL57, λ WL58. Preparation of this derivative without loss of restriction targets is described in M-16. Recombinant phages were recovered from WL95 (rk−mk+(P2)), pooled and infected at a m.o.i. of 0.1 onto a sup0 host (WL6). Phages will replicate and package their DNA but cannot lyse the cells. If recombinants are present which carry and express the restriction and modification genes, they will restrict a super-infecting lysis-proficient phage carrying targets for the appropriate enzyme. If the super-infecting phage is not restricted, it will lyse the cells. The infected sup0 host cells, containing the pooled recombinants, were therefore super-infected after 20' with an h80s+ phage (λ WL24). Surviving cells were harvested after 90', washed to get rid of phage in the supernatant and artificially lysed. The resulting
phage population should be enriched for the desired recombinant. After repeating this enrichment procedure three times, the phages were plated on a supF tonA host (WL5). Individual plaques were screened for their ability to complement an Eam Wam phage or to recombine in a cross with this phage (\(\lambda\)WL26). Failure to recombine or to complement, as compared to a control, would indicate restricting activity in the isolate. Several such attempts gave no recombinants with restricting properties. Crude extract tests (M.40) of infection experiments using pooled lysates of 10 recombinants each did not restrict \(\lambda\) DNA.

To investigate the possibility that phage carrying a gene for a restriction enzyme is not viable, we decided to use the PstI gene as a model system. The restriction and modification genes of Providencia stuartii 164 had recently been cloned on a 4 kb HindIII fragment in plasmid vector pBR322 (Walder et al., 1981). I decided to repeat this experiment (though not using their elaborate procedure) and transfer the Pst genes into \(\lambda\) to study the behaviour of the resulting phage.

b) Cloning the PstI restriction and modification genes into pBR322

DNA from Providencia stuartii 164 and pBR322 was restricted with HindIII mixed and ligated (see fig. 6-1).

After transformation in WLO6 (rk^-mk^+) cells were grown in 1 ml LB for 1 hr at 30°C. The cells were then grown in 10 ml LB+Mg+amp (100 µg/ml) at 37°C for several hours. Fresh amp was added after the first 2 hours. The cells were spun, resuspended in 1 ml LB+Mg and phage were adsorbed at m.o.i. 5 for selection of recombinants. Cells were incubated overnight on L+amp plates. Large survivors were purified and checked further. Five isolates showed restriction of a series of different lambdoid phages. \(h^c\) (\(\lambda\)WL73) was restricted with an efficiency of \(10^9\). Surviving plaques from any of the 5 strains were resistant to further restriction by the other strains,
indicating the same restriction and modification system being present in all 5 strains. These results are shown in table 6-1. WL98, the isolate which was used in most subsequent experiments, is compared with the other isolates, WL99-WL102 (see table 6-1).

WL98-102 were tested in a crude extract test (M-C) and showed a digestion pattern of λ c DNA consistent with a partial digest with purified PstI from a commercial source.

DNA of the plasmids in these strains showed, upon digestion with HindIII, a band comigrating with pBR322 and one of approximately 4 kb, which was consistent with the data of Walder et al (1981). There are no EcoRI or BamHI targets on the cloned fragment.
Table 6-1 Properties of WL98-WL102 (37°C)

<table>
<thead>
<tr>
<th>Host</th>
<th>λ c.K **</th>
<th>λ c.98 ***</th>
<th>λ c.99-102 ***</th>
<th>Titre λWL73</th>
</tr>
</thead>
<tbody>
<tr>
<td>WL98</td>
<td>p -</td>
<td>+</td>
<td>+</td>
<td>2.10^2</td>
</tr>
<tr>
<td>WL99-102</td>
<td>p -</td>
<td>+</td>
<td>+</td>
<td>2.10^2</td>
</tr>
<tr>
<td>C600</td>
<td>+ +</td>
<td>+</td>
<td>+</td>
<td>9.10^11</td>
</tr>
</tbody>
</table>

* phages with hostrange λ and φ80 and immunity regions of λ, 21 and λ34 were tested: all tests were carried out by spotting dilutions on the bacterial lawn; p = single plaques in the spot; - = no plaques; + = confluent lysis in the spot.

** phage was tested at 10^11 and 10^10 φ/ml.

*** phage was tested at 10^5-10^9 φ/ml.

Conclusion: WL98-102 carry a restriction and modification system which is the same in all five strains. These strains restrict λ.K with an efficiency of ca. 10^9.

c) Transfer of the Pst-genes into pL-asmid

The strain carrying the Pst-genes on pBR322, WL98, gives a high yield of the PstI enzyme. According to Walder et al the yield is 10 times that of Providencia itself. To test whether a further improvement could be obtained, I transferred these genes from pWL98 into pL-asmid, pWL107, a plasmid carrying the strong λ promoter for leftward transcription, P_L, under control of the cl-gene. It carries the (sbhI λL-sri λL) fragment between the EcoRI and BamHI sites of pBR322, inactivating the tet-gene (fig.6-2).

It carries a BglII-generated deletion, which removes the cro-gene. This
plasmid will therefore allow continuous expression from $p_L$ when the $cI$ repressor is inactivated. Cloning into the BamHI site, downstream of $p_L$, should give high yields of product if the fragment is in the appropriate orientation. A partial digest of pWL98 DNA with MboI was mixed with a BamHI digest of pWL107 DNA and ligated. After transformation and selection for $amp^R$ cells, the culture was challenged with $h^{80 imm21c}$ and $h^\lambda c_m$ (i.e. $h^\lambda c$ which has been previously modified against restriction by PstI by growth on WL98). The first phage will kill the cells harboring pWL107, the second will eliminate those carrying pWL98. Five survivors which were immune to $\lambda$ at $32^\circ C$ and carry the Pst-genes were found (WL108-112). Their properties are listed in table 6-II.

The restriction efficiency of these strains at $32^\circ C$ was $10^9$, like that of WL98.

At $37^\circ C$ or $42^\circ C$ WL108-112 were not viable.

Crude extract tests comparing restriction activity of WL108 and WL110 at
### Table 6-II  Properties of WL108-WL112 (32°C)

<table>
<thead>
<tr>
<th>Phage</th>
<th>Host</th>
<th>λc.K</th>
<th>λc.98</th>
<th>λc.109-112</th>
<th>Titre of λWL57</th>
<th>Phage λimmλ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WL98</td>
<td>p</td>
<td>+</td>
<td>+</td>
<td>10^2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>WL108</td>
<td>p</td>
<td>+</td>
<td>+</td>
<td>10^2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>WL109-112</td>
<td>p</td>
<td>+</td>
<td>+</td>
<td>10^2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C600</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>10^{11}</td>
<td>+</td>
</tr>
</tbody>
</table>

* phage carrying imm21 or imm434; the behaviour of λimmλ phage is illustrated in the last column for himm c after modification of the DNA on WL98 (φWL75 = φWL73m).

The experiment was carried out as described in table 6-I.

Conclusion: WL108-112 are immune to λ at 32°C and carry the λ restriction and modification genes. These are expressed at 32°C and therefore expressed from a promoter which is not P_L, possibly the same promoter as in pWL98.

32°C and 37°C with WL98, showed no difference in activity up to an eight-fold dilution of the extract (DNA analysis showed different restriction patterns for WL108 and WL110, while WL109, WL111 and WL112 gave patterns similar to that of WL108). Whether the lethal effect of the high temperature on WL108 and WL110 obscures a higher level of enzyme production in the induced cells has not been further investigated (see discussion).

d) Transfer of the 4 kb HindIII fragment, carrying the λ restriction genes into λ

The 4 kb HindIII-generated fragment of pWL98 carrying the λ restriction genes was transferred into a HindIII vector. Since λWL57 requires a minimum insert size of about 7 kb, λ NM540 (λ WL84), λ (sr1 λ 1-2) imm21 (nin5) shn λ 6°,
was used.

1) Cloning the Pst genes into λ NM540.

DNA from pWL98 and this phage was restricted with HindIII (fig. 6-3).

After ligation and packaging, the phages were adsorbed onto QR47 and grown for 1 hr at 37°C in LB+Mg. 0.1 ml of this culture was mixed with 0.2 ml of fresh WL98 cells, plated on L-plates and incubated at 37°C. Two types of plaques were found: fuzzy ones, parental phage which grew on the QR47 cells but were restricted by WL98, and clear ones, growing on both hosts. Some of the latter were purified on WL98 to give λ WL77 isolates. These phages gave clear plaques on WL98. Other turbid phages (e.g. λ WL9) also plated as clears on this host.

2) Properties of λ WL77 isolates, λ Pst imm21

Plate lysates of 4 isolates of λ WL77 showed that the progeny were clearish instead of turbid like the parental phage (λ NM540) on hosts other than WL98 (e.g. Ymel, QR47, C600 and Φ3350), though there were many turbid phages present in the lysates. The clear plaques in these lysates
threw off turbid plaques at high frequency; this frequency varied and was higher on e.g. Ymel than on \( \nu^350 \).

Windass and Brammar (1979) showed that \( \lambda \text{imm}21 \) phages carrying a plasmid origin of replication of a high copy number plasmid have clear plaques. They showed this to be due to the fact that replication from the plasmid origin proceeds so rapidly, that it titrates the 21 repressor. To check whether \( \lambda \text{WL77} \) isolates carried this origin (i.e. they carry the whole pWL93 plasmid instead of the 4 kb fragment) the phages were spottested on a host lysogenic for \( \lambda \text{imm}21 \). \( \lambda \text{WL77} \) did not plate on such a lysogen and it was therefore concluded that the clearish phenotype was not due to the presence of a plasmid origin.

The turbid and clear phages in the lysates were checked for modification by spotplating on WL98 and C600. The turbid plaques proved to have lost their ability to modify, failing to plate on WL98, while the clearish plaques did grow on WL98, though with low e.o.p. \( (10^{-4} - 10^{-5}) \). Our explanation for this is that the modification enzyme cannot keep up with the fast rate of replication of the phage DNA. After many attempts one lysate of \( \lambda \text{WL77} \) isolate 2 was obtained on \( \nu^350 \), which contained only a small proportion of turbid plaques.

The properties of \( \lambda \text{WL77} \) isolate 2 are listed in table 6-III and compared with \( \lambda \text{c} \) (p. 6-9).

We surmised that the high loss of the fragment was due to overproduction of the restriction enzyme. This proved later to be incorrect.

e) Preparation of a heat-inducible lysogen carrying the Pst-genes

1) Introduction of \( \text{cl857 Qam} \) into \( \lambda \text{WL77} \)

The right hand part of the \( \lambda \text{WL77} \) genome was replaced by one carrying a temperature-sensitive repressor gene and a Qam mutation, which should enable us to obtain a good yield of enzyme and might be more stable than the lytic phage.
Table 6-III
Properties of $\lambda$WL77: $\lambda$ Pst imm21

<table>
<thead>
<tr>
<th>Phage</th>
<th>C600</th>
<th>WL98</th>
<th>C600</th>
<th>WL98</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda$ c.98</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>(c)</td>
</tr>
<tr>
<td>$\lambda$WL77.98</td>
<td>+(c)</td>
<td>+(c)</td>
<td>+(c)</td>
<td>+(c)</td>
</tr>
<tr>
<td></td>
<td>+(tu)</td>
<td>-</td>
<td>+(tu)</td>
<td>-</td>
</tr>
</tbody>
</table>

A plaque of $\lambda$WL77 was picked from WL98 and spotted on C600. Plaques on C600 (c and tu) were checked for ability to modify their own DNA by spot-plating on WL98; $\lambda$WL75 ($\lambda$imm $c_m$) was used as a control.

- = no plaques in the spot; + = confluent lysis in the spot;
+ = single plaques in the spot.

Conclusion: $\lambda$WL77 clear plaques express the modification gene of PstI, though modification is not complete; $\lambda$WL77 turbid plaques have lost the ability to modify their own DNA.

For this cross $h^{-80} trp51 cI857 Qam73$ was first modified against PstI by making a lysate on WL98 ($\lambda$ WL80). $\lambda$ WL77 was then crossed with this phage on QR47:

\[\text{(srl} \lambda 1-2)^{\Delta Pst} \rightarrow (\text{nin} 5)^{\Delta} \lambda WL77 \]

\[\lambda WL80 \]

\[\rightarrow \lambda WL77 \]

Cross 6.1: $\lambda$WL77 x $\lambda$WL80

Recombinants were isolated on C600 ( $\lambda$imm21)(WL32), purified on C600 and screened on WL98 and sup$^0$ for modification, indicating the presence of the
Pst-fragment and Qam. Again the preparation of lysates of these phages (WL81 isolates) without loss of the fragment was difficult. In this case loss of the fragment was measured by spotplating on WL96 and WL98. WL96 is the parental strain of WL98, lacking the plasmid. Plaques which had lost the Pst-fragment were larger on WL96 than those still containing the fragment. These results are shown in table 6-IV.

Table 6-IV  Growth of \( \lambda \)WL81: \( \lambda \) Pst cI857 Qam

<table>
<thead>
<tr>
<th>Host</th>
<th>Phage</th>
<th>WL96</th>
<th>WL98</th>
<th>WL96</th>
<th>WL98</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \lambda ) c.98</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>( \lambda ) WL81.98</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Tests were carried out as described in table 6-III.

s = small plaque; 1 = large plaque

Conclusion: The behaviour of \( \lambda \)WL81 is similar to that of \( \lambda \) WL77.

2) Properties of \( \lambda \) WL81, \( \lambda \) Pst cI857 Qam

Three lysates were obtained with a reasonable low proportion of revertants. They were checked in a cross for ability to restrict in vivo. There was no detectable difference in the number of recombinants between \( \lambda \)WL81 x \( \lambda \) WL57 and \( \lambda \)WL81 x \( \lambda \) WL57m (= \( \lambda \)WL78). Both crosses were done in OR47. Expression of restriction activity from \( p_L \) was therefore unlikely, since we would in that case expect a high level of enzyme to be present and to be detectable by its effects in a phage cross. The lack of detectable in vivo restriction could be due to low level expression of the restriction gene in the phage, a (too) high proportion of phages without the fragment present in the lysate, or to a rate of modification of the incoming DNA of \( \lambda \)WL57 sufficient to give no detectable reduction in the recombination
frequency.

3) Lysogens of $\lambda$WL81, $\lambda_{Pet}$ cI857 Qam

A lysogen containing $\lambda$WL81 was prepared in a sup° host at 32°C. Low level expression of the restriction gene from its own promoter should be detectable. After screening many colonies, I finally isolated one lysogen, (WL115), but this showed no detectable restriction or modification activity. This inability to find a lysogen with a Pet-insert would fit with the clear phenotype of $\lambda$WL77 and could be explained by damage to the host's DNA by the restriction enzyme carried on the fragment. If this is true, we should be able to make such lysogens in a modified host, i.e. WL98. We could then remove the plasmid and look at the phenotype of the lysogen. Since WL98 carries supE, $\lambda$ WL81 will be phenotypically Q+. pWL98 was therefore introduced into a sup° host, which was also deficient in endonuclease I (WL114) to give WL117: sup° endol⁻ (pWL98). It was later discovered that this strain carried an uncharacterised prophage, which, however, did not invalidate the data (another lysogen was later prepared in a strain lacking this prophage and showed similar characteristics). Two lysogens of WL117 were isolated (WL120 and WL121). It proved however to be difficult to eliminate the plasmid. Only after repeated growth in LB without amp for several days, two isolates of WL120 were found which had lost the plasmid, WL122 isolates 1 and 9.

4) Properties of lysogens of $\lambda$WL81

The lysogens WL122 isolates 1 and 9 showed in vivo modification at 32°C and 37°C, but no restriction. $\lambda$ c.122 plated with high efficiency on WL98, but modification is not complete. This is probably due to the presence of only a single copy of the modification gene, which gives too little modification enzyme to modify fast replicating phage.

Another strain, WL154, a lysogen of $\lambda$WL81 in the suppressing host HB101 (WL125) (supE recA) was checked in two ways: the induced lysogen
was spot tested on WL98 and WL96 and was fully modified. An \( h^{80, \text{imm21c}} \) phage was spot tested on WL154 at 32°C and plated with an e.o.p. of 1, indicating no restriction. Phages were tested subsequently on WL166 and WL167, \( \lambda^R \) derivatives of WL98 and WL96. These phages were partially modified and plated with an e.o.p. of \( 1 \times 10^{-2} \) on WL166 as compared to WL167. These data are summarised in table 6-V.

<table>
<thead>
<tr>
<th>Phage</th>
<th>Host</th>
<th>WL96</th>
<th>WL98</th>
<th>WL154</th>
<th>WL166</th>
<th>WL167</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\lambda \text{WL81})^i)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(\lambda h^{80, \text{imm21.K}})</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>(\lambda h^{80, \text{imm21.154}})</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+(10^{-2}))</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>(\lambda h^{80, \text{imm2.K}})</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

\* (\(\lambda \text{WL81})^i\) = induced lysogen of \(\lambda \text{Pst cI857 Qam}\) (host: HB101)

\** WL166 = \(\lambda^R \text{WL98}\)

\*** WL167 = \(\lambda^R \text{WL96}\)

Conclusion: WL154 shows modification, though this is not complete, but no detectable restriction activity at 32°C.

\( h^{80, \text{imm21}} \) was also adsorbed to WL122 at 37°C and plated on a \(\lambda^R\) host (\(\lambda^R \text{Q47 (WL82)}\)). As controls WL115 (an \(\text{amp}^8\) lysogen of \(\lambda \text{WL81}\) in WL114 which showed neither restriction nor modification) and WL120 (an \(\text{amp}^R\) lysogen of \(\lambda \text{WL81}\) in WL114, expressing full restriction and modification from the plasmid) were used. No plaques were found on WL82 after transmission through WL120, while \( h^{80} \) phage did come out of WL115 and WL122, showing that at 37°C there is also no detectable restriction in WL122. This supports our previous conclusion that the restriction gene is not expressed from \(\text{P_L}\). These data are summarised in table 6-VI.
Table 6-VI Properties of WL122 (sup^endo1^- (λWL31)) at 37°C

Unmodified phage was adsorbed to WL122 and control strains and the infected culture checked for the production of phage, see text for details.

Phage | λ^R_{OR47} (WL82) | restriction
---|---|---
\h^80_{h} imm21/122(37°C) | + | -
\h^80_{h} imm21/115(37°C) | + | -
\h^80_{h} imm21/120(37°C) | - | +

Conclusion: transmission through WL122 of \h^80_{h} imm21 is similar to that of transmission through a restrictionless control; WL122 therefore does not restrict in vivo at 37°C.

Crude extract tests at 32°C and 37°C of the induced lysogen of WL122 showed no detectable restriction pattern. So WL122 does not seem to restrict in vitro either.

5) The Pat-fragment in λWL81

It had proved impossible to make DNA preparations of λWL77 and λWL81 due to the high loss of the Pat-fragment. DNA was prepared from the induced lysogen of WL120. This showed the phage to carry a 4 kb fragment after digestion with HindIII, which comigrated with the insert of pWL98.

Transfer of this fragment into plasmid vector pAT153, a smaller derivative of pBR322, gave transformants which were indistinguishable from WL98, showing full restriction and modification activity (WL145, isolates 1-8).

This proved that it is lack of a suitable promoter in the phage or the lysogen, rather than a small deletion or point-mutation inactivating the restriction gene but not the modification gene, which causes the restrictionless phenotype of λWL77 and λWL31. This leads to the conclusion that the 4 kb HindIII fragment from Providencia carries the restriction gene, but not its promoter, while the modification gene is expressed from its own promoter. It also means that the restriction gene in the phage has to be read rightwards since there is no detectable expression from pL. There is no rightward promoter from which the restriction gene could be read, since it
is inserted at shn3, just to the right of a stop signal for rightward transcription at about 56% on the λ map. The rightward promoter \( p_{att} \) which is discussed in chapter 3, lies to the right of the insert, so cannot be used to express Pst.

This leaves us with the puzzling question why the fragment is lost from the population at such a high frequency, if the restriction enzyme is not expressed, even in a host carrying the plasmid pWL98. It suggests a possible negative effect of the modification gene on the growth of the phage.

The final conclusion is that the Pst restriction gene in pWL98 is expressed from a plasmid promoter. Since HindIII cuts at the start of the tet-gene we wondered if a promoter had been reconstituted using combinations of sequences from the tet- and Pst-genes (see fig. 6-4).

\[ f \] Expression of restriction in the plasmids carrying the Pst-genes

1) A reconstituted tet-promoter

If the restriction gene in the plasmids pWL98-pWL102 is expressed from a reconstituted tet-promoter, we should be able to express restriction in a phage by cloning the EcoRI-BamHI fragment covering the 4 kb HindIII-generated Pst-fragment (see fig. 6-4) from the plasmid into the phage.

Since there was no more DNA of pWL98 available, DNA of pWL100 was used (see section b)). \( \lambda \)WL57 DNA was partially digested with BamHI, mixed with a complete EcoRI digest of the same phage DNA and a complete EcoRI-BamHI double digest of pWL100 DNA. In this way I hoped to clone the Pst-genes between sr1\( \lambda \)2 and sbh1\( \lambda \)3, with the restriction gene expressed from the plasmid promoter and not from \( p_L \). Resultant phages showed no detectable in vivo (cross) or in vitro (crude extract test) restriction activity.

An EcoRI shotgun cloning experiment of the whole plasmid into the immunity region of EcoRI insertion vector \( \lambda \)WL9, b538 imm434, also gave no detectable
Expression of the PstI restriction and modification genes in plasmid pWL93 (and sister plasmids pWL99-pWL102; possibly for pWL108-112 as well).

2) A backwards reading amp promoter

A second possibility for the expression of restriction is a promoter in or near the amp-gene of pBR322, which reads clockwise through the EcoRI-site (see fig. 6-3).

To test this possibility a partial HindIII digest of pWL99 DNA was mixed with a total digest of \( \lambda \)NM540 DNA and ligated (note: at this stage I assumed all 5 isolates WL98-WL102 to express the restriction gene from the same plasmid promoter). Recombinant phages (\( \lambda \)WL91) were isolated as before (section d). Since these phages again tended to lose the fragment at a high frequency, lysosomes were made using single plaques after a
single purification step on WL98 (the plaques were shown to be mod\(^+\)). Some of the lysogens should show continuous expression of restriction and modification, consistent with one copy/genome.

The four possible phage recombinants from the above ligation are shown in fig. 6-5.

**Fig. 6-5:** the four possible combinations from the ligation pWL99/λ\(^{W540}\) (HindIII partial digest), leading to four different phage recombinants (\(\lambda\)WL01 isolates 1-3).
Isolates $a$ and $c$ would be unable to express the restriction gene from either phage or internal **amp** promoter as a lysogen ($c$ could express this gene as a lytic phage from $P_L$ of phage 21).

Isolates $b$ and $d$ could use the internal **amp** promoter for the expression of the restriction gene.

I decided to use strain WL108 for the lysogenisation. This strain carries the Pst-genes on pL-asmid and is not viable at 37°C (see section c). Since $\lambda$WL91 does not have a **ts** repressor gene, we can eliminate the plasmid (which is necessary for modification of the host DNA during the establishment of the lysogen (see section e)) by a temperature-shift.

The lysogen can not be induced since it is not temperature-sensitive and only cells which have lost the plasmid will survive (a small proportion might conceivably have lost not the whole plasmid but only part of it).

We can thus avoid having to grow the lysogens repeatedly in medium without **amp** and screening many isolates for loss of the plasmid as was necessary to obtain WL122 and WL154.

Phages recovered from purified plaques of 8 isolates of $\lambda$WL91 were adsorbed to WL108 and grown at 32°C for several hours in LB+Mg+**amp**. The cells were then washed to remove the ampicillin and unadsorbed phage and grown ON in LB at 37°C. The cultures were challenged with phage ($\lambda$imm21) to select for lysogens.

12 possible lysogens of each of the 8 isolates of $\lambda$WL91 were checked for immunity. Those which did not plate $\lambda$imm21 were also resistant to $\lambda$imm21 (AWL1104), indicating a lysogen and not the presence of restriction as the reason for the resistance to this phage.

All isolates plated $\lambda$imm $\lambda c_m$ (WL75), indicating that they had lost at least the immunity region of the plasmid pWL108, if not the whole plasmid.

Two or three lysogens of each of the eight phage isolates were analysed further. A few of them showed no modification (e.g. isolate 12), but most did modify $\lambda c$. Modification was not complete, consistent with the results
of WL15\(_4\) indicating the presence of one copy of this gene in the cell. Some of the strains showed full restriction of \(\lambda\)WL57 (\(\lambda_{imm434c}\)), some no restriction and some partial restriction activity.

All but one (3\(_1\)) produced phage, when spotplated on QR47, most produced phage on WL98 (again an indication of modification). Some isolates were resistant to ampicillin, some sensitive. These results are listed in table 6-VII (p. 6-19).

Two isolates 5\(_1\) and 7\(_1\), which showed modification and partial or complete restriction were tested for levels of restriction and ampicillin resistance. As can be seen in table 6-VII, all isolates which are \(amp^R\), show a high restriction level, while all those which are \(amp^S\) restrict at low level or not at all. The ampicillin resistance proved to be chromosome-born like the modification properties: 7\(_1\) was resistant up to 50 \(\mu\)g/ml ampicillin at single colony level, whereas WL98 was resistant to 500 \(\mu\)g/ml. 5\(_1\) is sensitive to 1 \(\mu\)g/ml like QR47 under these conditions. These data are consistent with one copy/cell for isolates like 7\(_1\).

Restriction by 7\(_1\) was as for WL98 (ca. 10\(^9\)) while 5\(_1\) showed a ten-fold drop in plating efficiency of \(\lambda\)WL57. These results are listed in table 6-VIII (p. 6-19).

These puzzling results were explained by the fact that a high copy number plasmid cannot integrate into the chromosome unless:

a) the strain is polA

b) there is a second high copy number plasmid in the cell

c) the origin of the plasmid is deleted (Yamaguchi & Tomizawa, 1930).

By asking a phage carrying such a plasmid to integrate, we have a similar situation. Selection for lysogens (in a polA\(^+\) host), by challenging with \(\lambda_{imm21}\), and selection for loss of the plasmid (or part thereof), by raising the temperature, produces these rare lysogens carrying deletions covering the plasmid origin. These deletions are most likely to extend from the attachment site of \(\lambda\). The results can easily be explained from
Table 6-VII: Properties of lysogens of 8 isolates of \( \lambda \) WL91

\( \lambda \) WL96(\( \lambda \)imm21(pWL99))

| Restriction | Phage**
|-------------|-----------
| Isolate | \( \lambda \)imm21 | \( \lambda \)imm21<sub>m</sub> | \( \lambda \)imm434 | \( \lambda \)imm434<sub>m</sub>
| 1 2 1 8 | - | - | + | +
| 1 2 2 5 1 5 6 6 1 6 2 | - | - | + | +
| 3<sup>***</sup> | - | - | - | +
| 3 4 3 5 7 1 7 4 | - | - | - | +
| 4 2 | - | - | + | +

Modification/ampicillin resistance

<table>
<thead>
<tr>
<th>Isolate</th>
<th>mod.**</th>
<th>( \text{amp}^R )(20 ( \mu )g/ml)</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 1 8</td>
<td>+</td>
<td>-</td>
<td>( r^m ) ( \text{amp}^S )</td>
</tr>
<tr>
<td>1 2 2 5 1 5 6 6 1 6 2</td>
<td>+</td>
<td>-</td>
<td>( r^m ) ( \text{amp}^S )</td>
</tr>
<tr>
<td>3&lt;sup&gt;***&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>( r^m ) ( \text{amp}^R )</td>
</tr>
<tr>
<td>3 4 3 5 7 1 7 4</td>
<td>+</td>
<td>+</td>
<td>( r^m ) ( \text{amp}^R )</td>
</tr>
<tr>
<td>4 2</td>
<td>-</td>
<td>-</td>
<td>( r^m ) ( \text{amp}^S )</td>
</tr>
</tbody>
</table>

* \( \lambda \)imm\(^c\) gave similar results
** \( \lambda \)imm434\(^c\) was first plated on these strains and then checked on WL98 for modification; the lysogen was also checked for modification by checking the ability of spontaneously induced prophage to grow on WL98 (see text)
*** the only difference between 3<sub>1</sub> and 3<sub>4</sub> (etc) is that 3<sub>1</sub> carries a defective prophage; the lysogen could therefore only be checked for modification using the first method (see **)
**** phage was spotted at \(10^5 \text{ to } 10^9\) 6/ml

Table 6-VIII: Properties of two lysogens of \( \lambda \) WL91

<table>
<thead>
<tr>
<th>Strain</th>
<th>( \text{amp}^R )(( \mu )g/ml)</th>
<th>e.o.p. of ( \lambda ) WL97</th>
</tr>
</thead>
<tbody>
<tr>
<td>5&lt;sub&gt;1&lt;/sub&gt;</td>
<td>1</td>
<td>(10^{-1})</td>
</tr>
<tr>
<td>7&lt;sub&gt;1&lt;/sub&gt;</td>
<td>50</td>
<td>(10^{-9})</td>
</tr>
<tr>
<td>WL98</td>
<td>500</td>
<td>(10^{-9})</td>
</tr>
<tr>
<td>OR47</td>
<td>1</td>
<td>(10^0)</td>
</tr>
</tbody>
</table>
the 4 possible phages shown in fig. 6-5.
Isolate 42 is deleted for both the modification gene and the amp-gene. Isolates 1_1 (etc) have lost amp^R, do not express restriction, but have retained the modification gene and express this from its own promoter. Isolates 1_2 (etc) presumably have lost amp^R and now express restriction at low level from another promoter, e.g. a weak promoter in the tet-gene. Isolates 3 and 7 have retained the amp-gene and show full restriction activity, possibly from a promoter in or near the amp-gene as postulated. Isolate 3_1 has lost part of the phage DNA to the left of the shnλ3-site but it still carries the immunity region of phage 21, due to the selection used.
Selection for colonies immune to phage 21, has prevented us from isolating lysogens deleted for material to the right of the cloned plasmid, extending past the immunity region.

g) Conclusions and discussion
The results obtained with the restriction and modification genes of Providencia stuartii 164 in λ suggest that it is difficult to maintain these genes in λ even when there is no detectable in vivo or in vitro restriction activity. Despite this lack of restriction activity the Pst-fragment is readily lost from the phage population, suggesting a possible negative influence of modification on the propagation of phage carrying this gene, possibly via interference with the recombination or replication apparatus. The presence of the Pst-fragment in the phage reduced the rate of lysogenization, as judged from the clearish phenotype of such phages (λ WL77) on att^+ hosts. The frequency of lysogen formation seems also to be reduced in strains carrying the pWL98 plasmid. This is not only the case for λ WL77 but also for other turbid phages. This suggests a negative effect on integration functions also of the modification gene product.
Loss of the fragment occurs in all strains tested, though this is worse in e.g. Ymel than in B250. It also occurs in strains previously modified against *Pst*-restriction, i.e. strains harboring the pWL98 plasmid. The plasmid itself seems to be quite stable and is easily retained under drug selection without loss of the cloned fragment. Our original explanation for the fragment's instability in λWL77, observed as a tendency to revert from a clearish to a turbid phenotype on C600, was that the rate of expression of the restriction enzyme was so high or replication so fast that the modification enzyme could not keep up with it. However, if the rate of expression of the restriction was so high, we should be able to detect this activity in vivo in a cross or complementation test or in vitro. Neither of the two was the case. The results do show that the replication of the phage is too fast to completely modify the phage DNA, giving an e.o.p. of $10^{-4} - 10^{-5}$ on WL98 if the phage modifies its own DNA (λWL77 and λWL81 on C600) and $10^{-2}$ if the modification gene is expressed from a single copy in a lysogen (e.g. WL154).

The difference in modification level between the lysogen and the lytic phage could be simply that in the lysogen the enzyme is already present, while in the phage the enzyme will have to be produced first. On the other hand, the infecting phage will produce many copies of the gene, leading to more enzyme later on in infection, which could lead to a better modification level. In that case we explain the lower level of modification by assuming that the modification gene reads in the direction opposite to $P_L$, i.e. rightwards and is opposed by $P_L$ as has been described by Ward & Murray for trp-expression (chapter 3). Later on in infection the cro gene product switches off $P_L$ and we will get delayed expression of the modification gene. This idea could be tested. A cro- derivative of λWL77 or λWL81 should give very low or no modification. The problem is that there is no possible way of knowing that the fragment is present at all unless you make a lysogen: The phage should express no modification during lytic growth, but is expressing modification.
as a lysogen. The non-viability of the pWL108-112 strains at 37°C could be explained by such a model of convergent transcription in which \( P_L \) opposes the promoter of the modification gene. Since we have only two different isolates and have not established the orientation of the cloned fragment we do not know whether this is correct. Overproduction of the restriction enzyme could lead to cell death. However, there are reports that the expression of genes carried on pL-asmid can be much lower than expected. The cells often look feeble and die after prolonged incubation at 37°C. The possible explanation for this is that pL-asmid has no stop signal for transcription. Since the cro-mediated repressor has been removed, \( P_L \) is not switched off once the ci-repressor has been inactivated (cf. chapter 5).

Another explanation for the instability of \( \lambda WL77 \) would be leaky expression of the restriction gene through the stop signal at ca. 56% on the \( \lambda^+ \) map. The major efficient stop signal for rightward transcription in the b2-region has been removed by the (srI \( \lambda \) 1-2): we assume that the phage DNA, which is only partially modified, is sensitive to these low levels of restriction enzyme in the cell. This level is then however so low that it does not affect recombination frequencies and can not be detected in vitro which seems unlikely. As can be seen from the data on the lysogens of \( \lambda WL91 \) (e.g. isolate 5_1), we can easily detect a restriction activity which gives only a ten-fold reduction in plating efficiency.

Concluding, it seems likely that the modification gene is read from its own promoter. A clock-wise reading promoter in or near the amp gene seems a likely candidate for the expression of restriction gene in pWL99 and pWL100.

I assumed, that in the 5 plasmids WL98-WL102 the Pat-genes would be expressed from the same plasmid promoter. This however is not necessarily the case:

Since these experiments were carried out, Stüber & Bujard (1981) published their data on in vitro transcription patterns in pBR322 (see p. 6-23, fig.6) They find two partially overlapping promoters in the tetracyclin gene. \( P_2 \) is the promoter, from which the tet-gene is expressed, reading rightwards
Fig. 6.6: In vitro transcription patterns in pBR322
(data from Stüber & Bujard, 1981)

clockwise). $P_4$, just to the right of $P_2$, reads anti-clockwise through the EcoRI site into the ampicillin gene, which is also transcribed from $P_3$. $P_1$ and $P_2$ have partially overlapping RNA polymerase binding sites. The HindIII site lies just to the left of this overlap.

Near the origin two promoters give short in vitro transcripts: $P_4$ and $P_5$. $P_5$ is probably involved in the priming of DNA synthesis.

$P_4$ starts around 3 kb and reads clockwise; It is a very strong promoter in vitro, but gives a transcript of only a few hundred nucleotides long. Since transcription of inserts in the PstI site (in the amp-gene) which lack their own promoters is possible in both directions (and lead to the idea of an internal amp-promoter, which reads in the opposite direction), they postulated that this promoter produces a longer transcript in vivo. This promoter could be the one from which the restriction gene is transcribed in pWL99.

From the data of Stüber & Bujard it is possible that the restriction gene could also be transcribed in the opposite orientation from $P_1$, just to the right of the insert. Our data on pWL99 make it unlikely (assuming deletions most likely to have extended from the $\lambda$ attachment site) (see fig. 6-5) that
at least in this plasmid the restriction gene is expressed from $p_1$, since all $\text{amp}^R$ lysogens are $\text{r}^+\text{m}^+$, while all those which are $\text{amp}^S$, have lost partial or complete restriction activity. Were the $\text{Pst}$-genes expressed from $p_1$, we should have found lysogens of $\lambda$ WL91 which had lost ampicillin resistance, but still expressed full level restriction and modification, as well as ampicillin resistant colonies which had lost $\text{Pst}$-activity. Furthermore, we can explain the partial restriction of isolates like 51; the very weak expression of the restriction gene could be due to residual activity of the $\text{tet}$-promoter $p_2$.

High loss of the cloned fragment in the original isolates of $\lambda$ WL77 and $\lambda$ WL81 have prevented us from determining the orientation of the fragment with respect to that in the plasmid. It also made it impossible to try to invert that fragment with HindIII and see if restriction activity could be obtained by expression from $p_L$. Such a phage could however be even more unstable or even inviable.

Lack of time has prevented the preparation of fresh DNA of WL98-102 and of the 8 isolates of the cloning experiment of WL120i and pAT153 (WL145 isolates, see section e) to determine the orientation of the $4\text{ kb}$ insert in these plasmids.

In vitro expression experiments using the Zubay system (Zubay, 1973; Yang et al, 1980) should be able to give more insight into the various possibilities:

- does pWL108 and/or pWL110 express the restriction gene at $37^\circ\text{C}$, but not (or at low level) the modification gene? This would indicate that the modification gene is blocked by transcription from $p_L$, leading to cell death at $37^\circ\text{C}$: at $32^\circ\text{C}$ we should find two bands on the gel, corresponding with the two bands which are specific for pWL98 (according to Walder et al bands of 32000 and 35000 D); at $37^\circ\text{C}$ one of these bands would disappear. If the cause of cell death is inherent to the plasmid, the two bands will remain.
does pWL99 DNA, restricted with an enzyme such as BamHI or PvuII with
targets to the right of the insert produce two bands on the gel, while
restriction with EcoRI or HindIII leads to the disappearance of one band?
This would support the idea of a promoter in or near amp (p4?) for the
expression of restriction in this plasmid and the lysogens of pWL91.
do the other plasmids express the restriction gene from the same promoter?
Or can they also be expressed from p1?

One might conceivably expect (Walder et al.,81) the restriction and
modification genes in Providencia stuartii 164 to be expressed from the
same promoter. It is however possible that the modification gene can be
read from two different promoters. One is the promoter present on the 4 kb
HindIII fragment, the other one is the promoter for both restriction and
modification which is not present on the cloned fragment. Our data are
consistent, though no proof, of a model in which the modification gene is
located downstream of the restriction gene, both reading in the same
direction (the restriction gene in the phage is not read from a phage
promoter, and is therefore probably read in the rightward direction; the
rate of modification of phage DNA by phage-born modification genes is lower
instead of higher than that by a single copy in the bacterial chromosome,
which might suggest delayed transcription of these genes in the phage by
opposing transcription from p1; in that case the modification gene is read
from the rightward strand as well). Such a system would allow fine tuning
of levels of modification enzyme which is vital to the cell.

The results obtained with PstI can amply explain our failure to isolate the
genes for HindIII, BamHI and SalI in our original approach, if these phages
were to be as unstable as pWL77. Several enrichment cycles should be enough
to lose all recombinants which might be carrying these genes.
Discussion

The vectors \(\lambda W L 4 1\) and \(\lambda W L 5 7\) were designed as multipurpose vectors for use with a series of different enzymes, which would make them particularly suitable for the establishment of gene banks.

A powerful selection for recombinants makes it possible to use the vectors in a simple 'shotgun' cloning experiment. DNA from vector and donor are cut with the appropriate enzyme(s) and mixed for ligation. The recombinants can be selected on a strain lysogenic for P2. Part of the packageable DNA from such a cloning experiment consists of reconstituted vector molecules, with the dispensable central fragment in the normal orientation or inserted in the opposite direction (inverted orientation).

We have shown that such vector molecules, upon subsequent packaging and infection, do express the \text{red} and \text{gam} genes, which prevents subsequent growth of the vector on a (P2) lysogen.

Expression of the \text{red} and \text{gam} genes on an inverted \text{HindIII}- or \text{EcoRI}-generated fragment was expected, since in that case we invert the whole \text{N}-operon, including the promoter \(P_L\). Inversions of the central fragment with \text{BamHI} splits the \text{N}-operon, however, leaving the \text{N}-gene under control of \(P_L\) but expression of the \text{red} and \text{gam} genes will now have to take place from a different promoter in the rightward direction. We expected these genes to be read from \(P_R\)'.' A complementation test showed however \(Q\)-independent expression of these genes, which in view of later evidence of the existence of a strong \(Q\)-independent stopsignal for rightward transcription around 56\% on the \(\lambda^+\) map (Burt, 1931) is not surprising. The promoter near \text{att}, described by Kravchenko et al (1979) is therefore a likely candidate as the promoter from which the \text{red} and \text{gam} genes are expressed.

Karn et al (1980) prepared a \text{BamHI} replacement vector in which they deleted
sbhl\lam^4 and introduced a new \textit{Bam}HI site on the other side of \textit{\text{\(d_L\)}}{L}, thereby allowing expression of the \textit{red} and \textit{gam} genes from \textit{\(d_L\)}{L}, irrespective of the orientation of the central fragment, a wise but in this case unnecessary precaution.

We have checked that all phages from a mixed infection of \textit{\(\lambda\)}WL57 and \textit{\(\lambda\)}WL56 (a \textit{Spi}^- recombinant of \textit{\(\lambda\)}WL57) in a \textit{(P2)} lysogen are phenotypically \textit{Spi}^- when individual plaques on that lysogen were tested. However, if we harvest the phage on such a plate as a pooled lysate of recombinants, some \textit{Spi}^+ (vector) phages are still present. A second cycle on a \textit{(P2)}-lysogen is necessary: on subsequent amplification of the recombinant phage on a host permissive to \textit{\(\lambda\)}WL57, these few vector molecules could quickly outgrow the more feeble recombinants, which have to rely on the \textit{chi}-site and \textit{E. coli}'s \textit{recBC} product for their recombination, instead of the phage's own (more efficient) \textit{red} and \textit{gam} genes. Such lysates should therefore always be checked for the presence of \textit{Spi}^+ phages.

This type of cloning experiment does not involve elaborate biochemical techniques and can be carried out in most laboratories.

On the other hand the vectors \textit{\(\lambda\)}WL41 and \textit{\(\lambda\)}WL57 can be used for highly efficient cloning experiments, e.g. when preparing a gene bank of a mammalian genome. To cover the whole genome of such an organism, one has to maximize the cloning efficiency. This involves separation of the vector arms (which are re-annealed after restriction) from the central fragment using a sucrose gradient. To prevent incorporation of several previously unlinked small target fragments in one vector molecule, a size selection of the donor DNA after restriction is necessary, using either sucrose gradient or gel separation techniques. This also increases the cloning efficiency since all recombinant phage DNA will be of packageable length. This method has been used successfully in Leicester using \textit{\(\lambda\)}WL41 and \textit{\(\lambda\)}WL57 by A. Jeffreys (pers. comm.).

Note: Barry Ely in our laboratory found a small proportion of \textit{Spi}^+ phage in the progeny of such a cloning experiment, which upon subsequent
amplification quickly starts to outgrow the recombinant phage. So, like above (after selection on a (P2) lysogen), the presence of Spi phage has to be carefully checked.

To isolate the desired recombinant from a gene bank, one can sometimes use a genetic selection for the gene(s) of interest (e.g. Struhl et al, 1976). If a radioactive probe is available, the desired recombinant can be characterised using the method described by Benton & Davis (1977), in which the DNA in the plaques on a plate is denatured, transferred to a filter and hybridised with this probe. In this method aspecific binding will produce a background level, which can be reduced by pre-hybridisation with denatured sonicated DNA, e.g. of E.coli origin or poly(rA).

To map restriction sites on the cloned fragment, find homologous sequences elsewhere in the genome or in related organisms, study sequences adjacent to the cloned fragment or study the relationship between mRNA and genomic DNA in eukaryotes to find intervening sequences or genomic rearrangements, one can use the Southern blot technique (Southern, 1975). This method has been used by Jeffreys & Flavell (1977 a & b) in their work on the rabbit p-globin gene region.

In both methods a stringent control of the hybridisation conditions to minimize aspecific binding is necessary. With the Southern blot method, in which aspecific binding is reduced using poly(A) and salmon sperm DNA, picogram amounts of DNA can be detected in the filterbound DNA. Minimal contamination therefore of the probe with other DNA, e.g. E.coli DNA, can obscure the results by non-specific binding. Such DNA can be present in the filterbound DNA or in the probe itself.

Purification of plasmid DNA takes place on the level of the DNA and a slight contamination with chromosomal DNA is difficult to avoid. Phage DNA on the other hand, is prepared from purified phage, which is separated from the chromosomal DNA prior to the isolation of the phage DNA. Use of a probe,
based on a λ vector, may therefore be sometimes preferred to a plasmid probe, despite the plasmid's advantages when carrying a small fragment.

The contaminating RNA can also be present in the filter-bound DNA. Restriction enzyme preparations are tested for purity only insofar as they are biochemically pure (i.e., give proper restriction and ligation) but can be contaminated with small amounts of the DNA, from which they have been prepared. So purified PstI, prepared from pWJ98, could still contain some plasmid or chromosomal (E.coli) DNA, which would act as specifically in the Southern blot experiments. This is one reason why the possibility of a heat-inducible lysogen expressing the Pst-genes to prepare the enzyme PstI has been investigated, since most probes are used in conjunction with plasmid vectors like pBR322 (A. Jeffreys, pers. comm.).

Poth vectors λWL41 and λWL57 and recombinants thereof have been grown in Rec' hosts to give titres from 1-2 x 10^10 for recombinants to 2 x 10^10-10^11/ml for the vectors. Not much thought has been given to the use of recombination-deficient hosts in conjunction with λ vectors. The instability of many plasmid recombinants when using plasmid vectors has forced people long since to use recA hosts to minimize rearrangements and deletions and stabilize the plasmid. Lack of reports on rearrangements in λ has made people continue to use Rec' hosts, especially since the phage yield on Rec' hosts is always poorer than on Rec'. However, when cloning highly repetitive sequences, this might well result in undesired genetic exchanges (deletions, duplications) especially if the recombinant phage is small.

The mechanism of occurrence of deletions and duplications still remains a puzzle. They arise in the absence of the recA protein of E.coli and there are often no obvious stretches of homologous sequences flanking such an area (illegitimate recombination; Franklin, 1971; Emmons et al, 1975). Once established, duplications are highly unstable in recA' hosts, probably via the mechanism described by Pellett et al (1971) (see fig. 2-2).
The 'polymerase slippage' theory (Ghosal & Saedler, 1978) could explain how short duplicated sequences occur, leading to duplications (or deletions, if such a loop is attacked by nucleases). DNA polymerase or ligase mutants of E. coli show an increased deletion frequency (e.g. deletions in the tonB region (Coukell & Yanofsky, 1970) in polA mutants of E. coli). Duplications and deletions occur also at an increased frequency after UV damage or damage by X-rays (Hill et al, 1969; Schwartz & Beckwith, 1969; see Franklin 1971). Cohen et al (1979) find deletions in plasmids carrying rDNA repeats both in Rec and recA hosts, though this is worse in Rec⁺. This suggests involvement of proteins other than recA in the formation of these deletions. We could explain these results via a complicated interaction of recombination and replication (and/or repair) enzymes on recA-mediated and/or replication-intermediate DNA structures analogous to λ red's dependence on either recA action or replication. As already mentioned (fig. I-4) the recE pathway was found to be recA independent (as opposed to the recBC and recF pathways). It was also shown to have a function analogous to λ red. Stahl et al (1974) showed λ red's dependence on replication in the absence of recA. Such a dependence is possible to show for λ, but would be very difficult to prove for the recF pathway. Thus the recF pathway might depend on replication intermediates in the absence of recA and illegitimate recombination might also rely on such intermediates for its action. This would explain the effects of UV damage and E. coli polA and lig mutants on the formation of duplications and deletions.

The data of Emmons et al (1975) show a possible involvement of chi in duplication formation either directly or indirectly. These authors proposed a transactive product in λ tdel133 to stimulate duplication formation in λ b221. If this transactive product is (a) chi-sequence(s) in the 050-right arm stimulating recombination in the homologous left part of the two phages (both of λ origin), we should find loss of this transactive activity when using a derivative of λ tdel133 with a left arm of 050 instead of λ (i.e.
a 300 deletion mutant of ca. 79kb), since then there will be no homologous DNA for chi to act upon (Stahl & Stahl, 1975).

Stahl & Stahl (1977) find no chi-activity in recA or recAB hosts. However, in this case they use a \( \lambda \text{rec}^+ \) phase. Red does not use chi-sites for its action. They give no data on a \( \lambda \text{rec}^- \) phase in the same hosts. If the idea is true that some recombination enzymes can use either recA-mediated DNA structures (which will be the majority in a recA host) or replication intermediates (the minority in Rec\(^+\)), we might find a residual chi-activity in a recA host, but not in a recA recBC host of a \( \lambda \text{rec}^- \) phase. \( \lambda \text{rec}^- \) uses its own \( \lambda \text{red} \) system, relying on such replication intermediates in the absence of recA rather than the recBC system, which functions poorly in the absence of recA, but uses both pathways (red and recBC) when recA is present. (That \( \lambda \) uses both recBC and red when recA is present, can be deduced from the data of Stahl & Stahl (1977), which show intermediate chi-activity when red and recBC are active and full chi-activity when recBC is used in the absence of red). The recA-dependence of chi might be the result of the dependence of recBC on recA-mediated structures rather than replication intermediates, while red and recF can use both.

Nigel Grinter in our laboratory studied the influence of a chi-site on a derivative of plasmid RP4. He found that a chi-site severely destabilises the plasmid, which effect disappears in a recA host. (He also found that chi works only in one direction. This orientation dependency was also found in \( \lambda \), where it was explained by the fact that packaging is directional (Feiss & Bublitz, 1975; Faulds et al, 1979). Chi acts asymmetrically (Stahl et al, 1980) and the hypothesis is that chi-mediated (forked) structures somehow get trapped during packaging, when in the one ("wrong") orientation and so its activity is only observed in the opposite orientation. In RP4 such directionality could be explained by the fact that replication is unidirectional). The instability of chi-plasmids could be explained by the occurrence of intermediate structures of two or more plasmids, preventing
normal segregation from taking place upon cell division (N. Grinter, pers. comm.).

Whatever the mechanism underlying rearrangements, it is obvious that the use of recombination deficient hosts at least reduces these genetic exchanges, to obtain a clone which is a true representation of the DNA in its natural environment. The presence of repeated sequences, χ-like sequences (which do not only occur naturally in the E. coli chromosome but have also been reported in other organisms, e.g. Herpes simplex virus (Umene & Enquist, 1971)) or areas prone to duplications (e.g. the pla region (Capage & Hill, 1979), the arr-genes (Charlier et al, 1979: see also Anderson & Roth, 1977, Starlinger, 1977 and Weissberg & Adhya, 1977)), which could well be present in most genomes, could all endanger the normal structure of the DNA. They may or may not arise independently from the normal recombination system, but will lead to greater instability in Rec* than in Rec– hosts.

Since λ has a packageable size limit, large duplications or deletions, as found in plasmids, are selected against, since such phage DNA molecules cannot be packaged to give viable phage.

The danger of reoccurrence of a duplication in the left arm of λWL57, as was found in λWL41a, is not imaginary: we have received reports from workers who find a decreased cloning efficiency, which seems due to a duplication, which prohibits packaging of phages carrying the larger donor fragments in the ligation mixture. By choosing a medium sized plaque for the preparation of a liquid lysate, we found noduplication in the left arm of λWL57 DNA (λWL41 plates as a larger plaque than λWL57). However, human nature being what it is, there is a natural tendency to choose a large plaque, which could well carry a duplication. Once there, such duplications are unstable and may result in many tandem repeats through the action of the recA system. If such repeats become longer than the length of the central fragment minus 2 kb (see chapter 3, p. 3–9), we endanger the selection for SpI– phages, since we
can religate left and right arms to get a packageable DNA molecule lacking either the central fragment or a donor fragment. We can easily solve this problem by choosing a medium-sized plaque and preparing the phage lysates on a recA host such as QR48 (titres of \( \lambda WL57 \) on QR48 are routinely ca. 2 x 10\(^{10} \) plaque/ml). We might however consider introducing two copies of the central fragment in the phage to increase the size of the vector (Rim et al, 1970). \( \lambda WL57 \) carrying two BamHI generated central fragments would be 95% \( \Lambda^+ \) length and therefore less prone to duplications. On the other hand, the chi-site may be used to its advantage to increase the viability of a small clone by increasing the length of the phage DNA through a duplication in the left arm, rather than in the cloned fragment.

The danger of duplication formation in the recombinant phages can be reduced by careful size-selection of the DNA to be cloned to obtain phages of 90-100% \( \Lambda^+ \) length.

Gene banks of higher organisms require 'safe' vectors and 'hosts' in compliance with the NIH or CGMP guidelines. \( \lambda Ch4A \), a double amber derivative of \( \lambda Ch4 \) (p.I- 9) and \( \lambda StWES \), a triple amber mutant of \( \lambda St \) (p.I- 8) meet these requirements and have been used in 'safe' hosts such as DP50supF (Elattner et al, 1977). These safe hosts are all recombination-proficient. In the course of my work in Leicester, I have tested hosts deficient in recBC, which were not very satisfactory to use since yields of both vectors and recombinants are low. Hosts deficient in recAB are very feeble and revert to a recombination-proficient phenotype at high frequency. As mentioned QR48 (recA) can be satisfactorily used for the preparation of vector DNA, but cannot be used for the preparation of SoI\(^-\) recombinants.

Double amber mutations could be easily introduced into \( \lambda WL57 \), or a second amber mutation into \( \lambda WL59 \) using the marker rescue technique (chapter 6, section a) to obtain a vector which would meet these safety requirements.
A recently published λgtVHS derivative, λgtVHS T5622, which carries two T3-fragments and allows easy selection of recombinants on a strain carrying plasmid colR, has not been approved as a safe vector (Davison et al, 1980). A new approach allowing rolling circle formation of Spi- phages in a recA background by supplying λram in trans on a plasmid, has proven to be an interesting possibility (when used in conjunction with a kil- mutation), which will however need some more study to obtain the right balance between the level of transcription on the plasmid carrying the ram gene and the viability of the cell. The ram gene does not completely inactivate the recBC product (Sakaki et al, 1973; Stahl & Stahl, 1977). Such a host is therefore less likely to revert to a Rec+ phenotype than the recA recBC hosts described above, especially as ram is not expressed during cell growth at 32°C.

At the moment the best approach for efficient cloning seems to be:
a) preparation of the vector DNA on a recA host, starting with a medium-sized plaque and not a large one;
b) careful removal of the central fragment on a sucrose gradient to get as little contamination as possible;
c) careful size selection of the donor DNA to obtain recombinant phage of 90-100% λ+ length.
d) propagation of these phages on recA(p(ram)) (a better version of WL217), recAB sbcA (WL57) or recBC (WL67) hosts. As soon as the gene of interest is located, it should preferably be transferred onto a λred+ram+ phage vector or a plasmid vector in a recA host.

During the course of this work, the construction of a vector containing synthetic linkers carrying sequences for a whole series of enzymes, flanking the replaceable region, was frequently discussed. One could consider the splI λ 1 or splIac sites on the left of this area and splIimm34, sbhI λ 4 or splI λ 3 on the right hand side of this fragment. Use of
Instead of *spl* would allow an additional 2.5 kb cloning space. Use of *srlimph3* would give a vector along the lines of λWLP7, producing "red gam" recombinants and could be considered if a useful recA*(gam)* strain is constructed as a good recombination deficient host for these recombinants. For safety reasons we might consider the presence of the *H* gene (the *H* gene will help to keep the phage in the lytic phase) and use the *sbhl* site. This could be used in conjunction with a KH54-deletion in the *cl* gene, removing the two *HindIII* targets in that gene. If we want to leave *gam* on the recombinant phage we have to use *srl*3, which would decrease the cloning capacity as well as invalidate our selection for Spi phage. In such a case we would have to remove the *sbhl* site and it might be advantageous to use a kil derivative (in view of our own results and the fact that *bio kil* grows better than *bio kil* (Greer, 1975).

The kil-gene function is something of an enigma. There is apparently no space on the λ map but it maps in the region of the *gam*-gene. Greer & Ausubel (1979) isolated a 16000 D polypeptide from λkil* infected cells, which was lacking in λkil* infected cells. Whether the kil function is really a separate protein or whether it is a property of a domain of the *gam* gene product, remains to be solved. The *gam* gene product is ca. twice the size of the presumed 'kil' protein, i.e. ca. 20000 D (Sakaki et al, 1973). It is conceivable that the *gam* product has more than one function in the cell and that those functions have separate domains. Mutations like kil ochre 355 could thus leave 'normal' *gam* functions intact (i.e. inhibition of recBC nuclease and stimulation of rolling-circle formation) but destroy a second nuclease function. Our findings that a plasmid carrying kil* is lethal in recA but not rec*, supports the idea of a nuclease function.

Hadjfield (1980) investigated the possibility of propagating λ as a plasmid by forcing it into a monomeric life cycle, which could be switched to the
lytic phage cycle when desired, and could lead to an alternative vector system. He found an effect of kil on both establishment and stability of such a plasmid which he called a 'phasmid': \( \Phi^+ \) and kil\(^+ \) have a positive effect on the establishment of the 'phasmid' but make such plasmids even less stable than they already are. His explanation for the instability is the very slow rate of replication of the plasmids. The presence of kil and \( S \) functions cause cell damage, slow down cell division (to allow repair). Once established the absence of kil and \( S \) is desirable. He isolated mutants in \( \lambda \)'s left arm, which seem to counteract the kil-activity in this respect (amp-mutants). Kil function might be involved in packaging by producing single-stranded nicks, upon which the ter enzyme, helped by the \( \lambda A \) gene product for specific recognition of the cos-sites, might act.

Honigman (1981) used the inviability of a kil plasmid at 37°C in a recA host to clone \( \Phi \)-independent stop signals into the BamHI site at 71.4\(^{\prime}\) on the \( \lambda^+ \) map (the construction of this cloning vector is described in Honigman et al, 1981). This plasmid, pHA10, differs from pWL165 in that it is carried on pBR322 instead of pAT153. The copy number of pAT153 is twice as high as that of pBR322, which might explain why pWL165 is inviable in a recA host even at 32°C, while pHA10 is apparently viable at 32°C in a recA background.

An analogous kil function is present in phage Mu and has been used to select for recombinants by insertional inactivation of the Mu kil gene (Schumann & Lörl, 1980).

The idea of supplying \( \lambda \) functions in trans is not new: helper phage has been used for defective transducing phage (e.g. dgal (Arber et al, 1957, Campbell, 1957)). One could conceivably construct a vector which uses all the necessary packaging functions provided by a plasmid or an (excision- and recombination-deficient) lysogen, i.e. a sort of in vivo packaging cosmid system. A phage carrying large deletions might be more stable than the cosmids seem to be in the hands of many people.
The problems encountered with the attempted cloning of the genes for restriction enzymes and their modification genes have been amply discussed in chapter 6. Our data suggest a possible negative influence of modification on DNA replication or recombination and make it unlikely that such genes can be easily maintained on a λ vector.

The complex interactions between vectors and hosts as discussed in this thesis stress the importance of knowledge of the behaviour of those vectors and hosts and the availability of easy-to-use host-vector systems, understandable to non-lambdologists or even non-geneticists. A whole range of different vectors, based on both viruses and plasmids, because of their different 'life style', is therefore essential. Lambda vectors carrying λ control functions but also large deletions in other essential areas, in conjunction with a host system supplying these missing functions in trans, might become a viable alternative to the cosmid system. The availability of synthetic linkers might lead to the development of a portable λ vector with appropriate sequences flanking the essential areas, analogous to the portable lac promoters, at present in use.

Well understood prokaryotic vector systems should help in the development of eukaryotic systems, which are so much more complex and might lead to hybrid vector systems, allowing propagation of the DNA in both pro- and eukaryotic cells.
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Appendix

Genetic and physical map of λ (data from Blattner, pers. comm., 1981)

Figure 1. Genetic and Physical Map of Lambda DNA. To provide a basic framework for the λ map 67 restriction enzyme cut sites were mapped on λ27 DNA. Cut sites, the left and right ends and 3 landmarks known from sequence were assigned numbers from 1 to 72. Beneath the figure is a tabulation of the positions for each site. The same sites, are shown to scale beneath the map (two-character abbreviation). EcoRI cut site 61, within the longest sequenced region, is defined as coordinate 40,000. Note the left end is not zero! The thickened portions represent regions for which DNA sequence is known.
The construction and use of $\lambda$ vectors in molecular cloning.

Abstract
A multipurpose vector was constructed which can be used for cloning DNA fragments of about 20 kb generated by restriction enzymes EcoRI, HindIII, BamHI and enzymes producing the same sticky ends (e.g. PstI, PstI, MboI and Sau3A). Recombinants can be selected by their SpI phenotype and their propagation is facilitated by the presence of a chi-site. This choice of enzymes simplifies the cloning procedure considerably and makes this vector particularly suitable for the establishment of gene banks. It can be used in simple cloning experiments, selecting recombinants on a (F2)lysogen as well as for highly efficient cloning after removal of the vector's central fragment and size selection of the donor DNA.

This vector can also be used as an expression vector when cloning into the vector's BamHI sites using the powerful $\lambda$ promoter for leftward transcription, $\beta_L$, and the phage's antiterminator function, $\gamma$. Recombination-deficient hosts for the propagation of recombinants have been investigated to minimize undesired genetic rearrangements.

The absence of restriction targets for several enzymes in the vector arms led to attempts to clone the genes for these enzymes and their modification enzymes. When these failed, the genes of Providencia stuartii 164 were used as a model system to study the behaviour of such genes when carried on a $\lambda$ vector. These genes turn out to be highly unstable in such a situation and experiments suggest a possible negative influence of the modification activity on host and viral functions.