Studies on Transduction by Bacteriophage P1

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TABLE OF CONTENTS

Chapter II Materials and Methods

Chapter IV Studies on packaging initiated by *pac* sites on the chromosome

Chapter V Studies on plasmid transduction by P1

Reference

Declaration

I declare that this thesis was composed solely by myself and the experiments presented here were all my own work, except where stated.

Haomin Huang

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Abstract

The original aim of this thesis was to address the origin of the abortive transduction protein (TPA), which appears to join the ends of transduced DNA and render it refractory to degradation by host nuclease and recombination with the chromosome. We planned to isolate abortively transduced DNA (ATD) from transduced cells, which should have TPA attached, in order to characterize it. To increase the packaging of transducing DNA and TPA, a *pac* site was inserted into the "donor" chromosome which was labeled with BU to make it heavy. CsCl equilibrium gradients were used to separate the transducing from infectious particles, and the ATD-TPA complex was purified from transduced cells. This method proved technically difficult and I did not succeed in purifying sufficient ATD-TPA to characterize. I developed an alternative method hoping to increase the yield. Exonuclease V (RecBCD) was used to remove linear DNA from transduced cells, which should include fragmented host DNA but leave ATD intact as it is circular. However, the ExoV did not work efficiently in crude cell lysates; and the method would need to be developed further to have any chance of success.

I decided instead to study P1 packaging of transducing DNA further. The *argB* gene was replaced with the P1 EcoRI-20 fragment, which contains *pac*. Transduction and UV irradiation showed that transduction frequencies of chromosomal markers close to *pac* were increased several hundred fold and could be further increased by UV irradiation, indicating that TPA was likely to be bound as normal. Southern blotting demonstrated that packaging is unidirectional; that increase in transduction frequency decreases with distance from *pac* and that the packaging of markers up to 30min from *pac* was increased. 4 further *pac* sites were placed at intervals on the chromosome. Strains containing different numbers of *pac* sites were analyzed by transduction, titration, and hybridization. Some novel phenotypes were observed. First, the time when cells start to lyse after P1 infection is delayed in multi-*pac*-containing strains, suggesting that in WT strains P1 starts packaging viral DNA earlier than in multi-*pac* strains, perhaps because the *pac* sites on the chromosome compete for recognition and cleavage by Pacase. Furthermore, strains which have more than 2 *pac* sites give very poor plate lysates, whose titers are 1000-fold lower than WT (or one-*pac*-containing strain). However transduction frequencies are greatly elevated. This suggests there is a limiting material, which could be used up when there are more than 2 *pac* sites on the chromosome, and that there is competition for it in the formation of transducing vs infectious phages. The Pacase enzyme is a likely candidate for this substance.

Abbreviations

ATP	Adenosine 5'-triphosphate
bp	Base pair
BSA	Bovine serum albumin
dATP	Deoxy ATP
DNA	Deoxyribonucleic acid
dNTP	Nucleotide 5'-triphosphate
E. coli	*Escherichia coli*
EDTA	Diaminoethanetetraacetic acid
KAN	Kanamycin
kb	Kilobase
kD	Kilodalton
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RBS	Ribosome binding site
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TEMED	N, N, N', N', - tetramethylethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
Ts	Temperature sensitive

Chapter I

Introduction

1.1. Bacteriophage P1 overview

1.1.1. Bacteriophage P1

Bacteriophage P1 is a double stranded DNA phage with a polyhedral head and tail (For reviews see, Yarmolinsky and Sternberg, 1988). Uniquely, three distinct sized P1 particles are produced at each burst. They are termed P1B (Big, width of head: about 85nm), P1S (Small, width of head: about 65nm) and P1M (Minute, width of head: about 47nm) respectively (Anderson and Walker, 1960; Walker and Anderson, 1970). The P1B virion contains the entire P1 genome and is infective at low m.o.i. P1S and P1M virions are too small to encapsidate the entire P1 genome and produce plaques only at a high m.o.i, presumably because individual P1S or P1M cannot encode all the proteins required to form a plaque (Walker *et al.*, 1979). Commonly, burst sizes of P1 in *E.coli* are about 100-200 pfu per bacterium (Mise and Arber, 1976; Takano and Ikeda, 1976). In a burst, the ratio of P1B to P1S varies depending on both host and phage. Normally, the relative abundance of P1B in a lysate is highest, about 83% (Walker *et al.*, 1979), although *vad* deleted P1 derivatives produce as many P1S as P1B particles (Iida and Arber, 1977, 1980). For simplicity, P1 particles refer to P1B for the remainder of this thesis, unless otherwise specified.

The DNA of plaque-forming particles is double-stranded and linear (Ikeda and Tomizawa, 1965a), It is about 100kb in length including a terminal redundancy of about 12% of the genome. Evidence shows that recombination between terminally redundant ends of viral DNA permit cyclization (Ikeda and Tomizawa, 1968). P1 DNA is also cyclically permuted, which is a consequence of "headful" packaging (see below).

In addition, in every lysate, about 0.3-2% of particles carry only bacterial DNA. These can carry DNA from any region of the chromosome of the donor on which the lysate was produced, to a recipient, in which the transduced DNA can be integrated, in a process called generalized transduction. Bacterial DNA is found in P1S particles as well, but only the DNA in P1B transducing particles

appears to be associated with a protein (Ikeda and Tomizawa, 1965b). The protein is not found in infective P1 particles. This topic will be discussed in detail later.

The P1 virion consists of one major P1 head protein of about 44kD; 14 other head proteins were revealed by SDS-PAGE (Walker and Walker, 1981). In addition, nine tail proteins have been identified. The molecular weights of the two major tail proteins are about 21.4kD and 72kD. Although the buoyant density of the plaque-forming particles (infective particles) varies amongst the three different classes of P1 particles, the buoyant density of the plaque-forming P1B particles is about 1.476 g/ml (Kondo and Mitsuhashi, 1964; Mise and Arber, 1976). The buoyant density of the DNA of infective particles is 1.706 g/ml with a G + C content of 46% (Ikeda and Tomizawa, 1965a).

1.1.2. Overview of the P1 life cycle

P1 requires Ca^{2+} to absorb to *E. coli* cells (Franklin, 1969). The reason for this is still unclear. Possibly, Ca^{2+} plays a role in the proper alignment of phage tail fibers and cell receptors. In addition, Ca^{2+} seems to play a role in stabilizing P1 in phage lysates, especially in the presence of chloroform (Yarmolinsky and Sternberg, review 1988). Because the original P1 isolates gave plaques 10^3-fold more efficiently on *E. coli* strains B, C, and W than on some substrains of *E. coli* K12, and 10^7-fold more efficiently than on other substrains of K12, a mutant P1k was isolated that has an efficiency of plating of about 1 on all sub-strains of K12 (Lennox, 1955). A mutant, P1kc, found in P1k gives a clearer plaque on substrains of K12 and a higher titre than P1k, usually between 10^{10} and 10^{11} pfu/ml (Swanstrom and Adams, 1951). The lysates made from P1kc when preserved over chloroform are quite stable. Therefore, P1kc became the common P1 of laboratory use and was used in this work.

1.1.2.1. Cyclization of injected P1 DNA

Within 5min after injection of P1 DNA into the cell, P1 DNA is circularized. This step is probably necessary to avoid attack from host nucleases, such as RecBCD, (Exonuclease V) (Goldmark and Linn, 1972; Muskavitch and Linn, 1982; Yu *et al.* 1998). Although, like phage lambda and T4, it has been

reported that P1 also encodes its own inhibitor of the RecBCD nuclease (Sakaki, 1974), the inhibition must be incomplete, since Sternberg and Coulby showed that the DNA end not destined to be packaged into P1 head after cleavage during packaging is degraded rapidly by at least two cellular nucleases, one of which is RecBCD (Sternberg and Coulby, 1987a).

The circularization process occurs by recombination between the ends of the infectious, terminally redundant DNA molecules, since linear P1 DNA is about 100kb, while circular prophage DNA is only 90kb, 10kb shorter than linear phage DNA (Ikeda and Tomizawa, 1968). So, what recombination systems are responsible for cyclization? The loxP-cre site-specific recombination system must be at least a main system, if not the only one, involved in this process. The following observations support this idea. First, it has been reported that P1 *cre* mutants lysogenize the *recA* bacteria 10- to 25-fold less efficiently than does a cre^+ phage, though it can lysogenize $recA^+$ strains as efficiently as does a cre^+ phage (Schulz *et al.*, 1983; Sternberg *et al.*, 1986). Secondly, Segev and Cohen (1981) analyzed the conversion of ^{3}H-labled P1 DNA to a closed circular form after injection and found that cyclization could occur in $recA^+$ strains, *recA* strains and both $recA^+$ and *recA* lysogens but did not occur if the host was treated with chloramphenicol or rifampicin before infection, suggesting that the host recombination system cannot work on cyclization solely. However, if the Cre proteins have been accumulated previously in the Chloramphenicol-treated cells before infection, the cyclization could be restored.

The *loxP* site is a 34-bp sequence that consists of two 13-bp inverted repeats separated by an 8-bp spacer region (Hoess *et al.*, 1982). Cre (cyclization recombination protein) is a 343aa protein (Sternberg *et al.*, 1986), which is encoded by *cre*, which is located 434 bp clockwise from loxP and is transcribed in the clockwise direction. Three weak promoters of *cre* have been found and it appears that they are active in P1 lysogens. We are not yet clear about how P1 regulates *cre* expression. It seems that the host methylation system plays a role here. This is based on the observation that one of the *cre* promoters, P*cre*1, is sensitive to methylation by the *E.coli* Dam protein and 5 times more active in a *dam* host than it is in a dam^+ host (Sternberg, 1985; Sternberg 1986). The mechanism of the lox-cre system has been well studied. Depending on the orientation of the spacer sequence of two *loxP* sites, the DNA between two sites will be either excised if the orientation of two sites is directly repeated or be inverted if two sites are in inverted orientation (Abremski *et al.*, 1983). To cyclize the

entire P1 genome, two *loxP* sites have to be located in the terminally redundant regions. Based on the observations that the packaging site (*pac*) is 5kb counter clockwise from *loxP* and the direction of packaging is from *pac* toward *loxP* (Bachi and Arber 1977; Sternberg and Coulby, 1987a) along with the observation that packaging proceeds for an average of about 3-4 headfuls on any one concatemer (Bachi and Arber, 1977), only one of every 3-4 P1DNA, generated from the first headful, contains *loxP* sites in the terminally redundant region and can be cyclized by the lox-cre system.

So far, alternative modes of cyclizing P1 DNA have not been found, though it has been shown that in addition to the lox-cre system, there should be other systems involved in cyclization of P1 DNA, since P1 *cre* mutants lysogenize *recA*$^+$ strains no less efficiently than does P1 wild type (Schultz *et al.*, 1983; Sternberg *et al.*, 1986). One possibility is that P1 perhaps encodes a protein that helps the host generalized recombination system to cyclize the injected P1 DNA. The evidence that the lysogenization frequency of *cre*$^+$ phages in a *recA* strain is reduced 2- to 5-fold relative to lysogenization of a *recA*$^+$ strain is consistent with this hypothesis (Segev and Cohen, 1981; Sternberg *et al.*, 1986). However, so far the P1 cyclization enhancement gene has not been identified. In addition, besides cyclization, the lox-cre site-specific recombination system appears to play an important role in other events of the P1 life cycle, such as the maintenance of the P1 plasmid state and the vegetative growth of the phage (Yarmolinsky and Sternberg, review, 1988).

1.1.2.2. Lysogenization of phage P1

Because a temperate bacteriophage, after P1 DNA is injected into the cells, circularizes by recombination and avoids attack of the restriction enzymes, P1 has to make a decision: either lysogenize the cell or undergo the vegetative (or lytic) cycle which results in cell lysis. If the conditions favor the prophage state, lysogeny will be established. Prophage P1 is an extrachromosomal replication unit (Ikeda and Tomizawa, 1968). The prophage P1 DNA is about 90kb. It exists in a circular form and is not physically associated with the bacterial chromosome; it therefore must replicate autonomously. Due to the above features which characterise plasmids, P1 prophage is also called P1 plasmid. The replication of P1 plasmid must be under tight control, since the average number of prophage P1 per bacterial chromosome is one. In addition, the P1 plasmid also appears to

be rather stably maintained since the curing frequency of resident P1 is about 2 x 10^{-5} cells per generation (Rosner, 1972). The molecular mechanism and genetic control of establishment of P1 lysogeny and prophage maintenance are still poorly understood. However, 3 regions that are involved in the immunity system which prevents superinfection and allows establishment of lysogeny, have been located at separate positions on the P1 genome. They are the ImmC region, where *cI*, the principal P1 repressor which represses the lytic functions by interacting with operator sites at several key promoters on the P1 genome (Eliason and Sternberg 1987; Velleman *et al.*, 1987), is encoded (Scott 1975, Scott 1980b), the ImmT region, which contains the *bof* gene involved in regulation of the activity of *cI* repressor (Sternberg and Hoess, 1983) and the ImmI region, which encodes an antirepressor gene *ant*, along with a repressor of *ant*, the *c4* gene (Scott, 1975; Wandersman and Yarmolinsky, 1977). The detailed picture of the mechanism of regulation of the P1 immunity system is not entirely clear. Work carried out on P1 replication revealed that the origins of P1 vegetative replication and P1 plasmid replication, termed *oriL* and *oriR* respectively, are independent of each other. This is supported by the following observations. First, P1cm mutants with deletions covering about 30% of the P1 genome, eliminate vegetative replication but leave plasmid replication unaffected (Austin *et al.*, 1978). Secondly, *recA* mutants have no effects on P1 plasmid replication, while sigma vegetative replication is diminished significantly in *recA* strains (Rosner, 1972). In addition, a *dnaA* null mutant blocks P1 plasmid replication, but not vegetative replication (Hansen and Yarmolinsky, 1986). The R-replicon, isolated by Sternberg and Austin (1983), is believed to be the plasmid maintenance replicon and it is not affected by *cI* and *c4* genes but appears to be *DnaA* protein-dependent (Hansen and Yarmolinsky, 1986). A phage-encoded protein, designated *repA*, is essential for the activity of the R replicon. It seems that P1 plasmid replication from *oriR* is under control of the products of *incA* and *repA*, since introduction of multicopy *incA* or incorporation of a *repA* mutation into P1 can interference with P1 plasmid replication (Chattoraj *et al.*, 1984; Yarmolinsky *et al.*, 1983). In addition, P1 also encodes it own partitioning functions. Three genes, *parA*, *parB* and *incB* appear to be involved (Sternberg and Hoess, 1983).

Once lysogeny is established, P1 expresses its own modification and restriction system to maintain the lysogeny. The P1 modification and restriction system allows P1 to protect itself from deleterious effects of foreign viral and cellular genes. The genes encoding modification (*mod*) and restriction (*res*) enzymes have been located about 4kb clockwise from loxP on the P1 map. The genes

are next to each other and *mod* is on the loxP-distal side of *res* (Sternberg, 1979; Heilmann *et al.*, 1980b). Although both the *res* and *mod* genes have their own promoters, it was found that *res* and *mod* may be transcribed together starting at the *mod* promoter. The P1 EcoP1 restriction enzyme is actually a type III restriction/modification system, consisting of two subunits of 106 – 110 and 73 – 77 KD, Res and Mod, (Heilmann *et al.*, 1980b; Hadi *et al.*, 1983). Previous studies (Iida *et al.*, 1983; Hadi *et al.*, 1983) indicated that the Mod subunit has both the modification and sequence-recognition functions of the enzyme, since no restriction function can be detected if the Mod subunit is inactivated, while Mod subunit can function to modify DNA in the absence of the Res subunit. The site that Mod recognises is a 5bp asymmetric sequence 5'-AGA*CC-3'. The enzyme methylates the adenine in the middle of the sequence. If an unmethylated 5bp site is found by the enzyme, the Res subunit cleaves the DNA 25-27bp 3' to the recognition site and leaves a 2-4bp 5' overhang (Bachi *et al.*, 1979).

Apparently, it is essential to ensure that Mod functions before Res does. This is supported by the fact that Mod function can be detected within 15 min after infection, while Res activity can only be detected 1h later and does not reach the maxium level until 3-4 h after infection. The control of this process is rather beautifully built. Studies on the ribosomal misreading of a stop codon (Arber and Dussoix, 1962; Engelberg-Kulka, 1979; Petrillo *et al.*, 1983) indicate that although both Res and Mod subunits are detectable early after infection, functional Res proteins appear much later than functional Mod proteins do. This is because the termination codon of the *res* gene is UGA and suppression of UGA by ribosomal misreading is crucial to produce a fully functional Res protein. Based on the above work, C. Levy, S. Iida and T. Bickle proposed that an inactive Res subunit which lacks restriction activities will be produced if translation stops at the UGA codon in *res*. Only if the UGA codon is suppressed and read through, will an active Res subunit be produced. Since the UGA codon is suppressed in the wild type cell about 1-2% of the time, the functional Mod protein would be produced 100-fold faster than would the functional Res protein (for review, Yarmolinsky and Sternberg, 1988).

1.1.2.3. Vegetative cycle of phage P1

If the circumstances do not favour establishment of P1 lysogeny, P1 will enter its vegetative cycle, resulting in cell lysis and production of P1 progeny phages. By the fifth minute after infection at 37°C, synthesis of phage DNA is observed and the rate will reach a level of about 95% that of total bacterial DNA synthesis after about 30min (Segev, N. *et al.* 1980). By isolation of replication intermediates from P1 infected cells and using electron microscopy, Cohen (1983) observed that at early times in infection, about 15-30min, replication intermediates are of both theta forms (θ) and sigma forms (σ). In lytically infected cells and at later times in infection, sigma molecules are the predominant replicating form. Interestingly, Cohen also found that sigma forms are very rarely seen in P1-infected *recA* cells, indicating that the bacterial *rec* recombination system might play a role in switching from theta replicating forms to sigma, possibly rolling circle replicating forms. It has long been known that P1 produces very poor lysates on *recA* strains. The finding that the *rec* recombination system is involved in P1 vegetative replication sheds light on this observation. This is supported by Segev and Cohen's (1981) finding. They found that during the first 15-30min after infection, closed circular DNA is produced at the same rate in both *recA* and $recA^+$ cells but that later, the accumulation of the circular DNA abruptly ceases in $recA^+$ cells, whereas it continues in *recA* cells. However, the total P1 DNA is about 10-fold higher in a $recA^+$ cell than it is in a *recA* cell, suggesting that DNA replication in $recA^+$ cells must still continue after 30min, but converts to other replicating forms, such as sigma forms. Furthermore, by examination of the concatemeric DNA from cells that contain two types of replicating phages, P1 and P1dlac, Bornhoeft and Stodolsky (1981) demonstrated that in the same concatemeric DNA molecule, only one type of phage is included, suggesting that the sigma forms are not formed by recombination between phage genomes but generated by rolling cycle replication. However, how the switch between the replication modes is triggered and the role of the *rec* recombination system in this process is still unclear.

As mentioned above, besides *oriR*, the origin of plasmid replication, another replicon, known as L (Sternberg and Hoess, 1983) was identified on a stable, low copy number miniP1 plasmid, pIH1972 (Shafferman *et al.*, 1978, 1979), which lacks both *oriR* and *repA*. Previous studies on the L replicon (Chattoraj *et al.*, 1985; M. Yarmolinsky *et al.*, 1989) demonstrated that the L replicon can take over the role of *oriR* in plasmid maintenance in some circumstances when *oriR* is prevented from

functioning. However, Yarmolinsky and Sternberg (review, 1988) think that the activity of the L replicon involved in P1 prophages is probably due to the leaking of immunity of the prophages studied, since all are derivatives of the relatively clear plaque mutant originally selected by Lennox (1955). This is supported by the observation that the leaky expression of *ref* at 30°C by P1Cm *c*1.100, which is possibly due to incomplete repression of *ref* by the thermosensitive *c*1 repressor, and a slightly increased plasmid copy number resulting from a modest activation of the L replicon, is eliminated by wild type *c*1 repressor but only slightly reduced by interrupting *oriL* with a Tn5 insertion (Windle, 1986).

Some evidence indicates that the L-replicon is the primary lytic replicon of P1. Two P1 lysogens with IS50 insertions in *Eco*RI-14 were isolated by Hansen (1989) using P1Cm*c*1.100E17::Tn5 (Windle, 1986). They appeared to be temperature-resistant. Thermal induction of the prophages does not trigger lytic DNA replication for at least 50min and cells do not lyse, although a slow release of phages could be detected (Hansen, 1989), suggesting that these temperature-resistant mutants lack the capacity for vegetative replication (for review, Yarmolinsky and Sternberg, 1988). In addition, the L-replicon is a high copy number replicon and can be repressed by *c*1 (Sternberg and Austin, 1983). The L-replicon is believed to be activated by a transcript from the *c*1-repressor-sensitive promoter P53 which is located in EcoRI-14 (Sternberg and Hoess, 1983; Sternberg *et al.*, 1986). Hansen (1989) showed that two IS50 insertions are both actually located within the above transcript, which is from an open reading frame of 266 codons, designated *kilA*. Expression of *kilA* gene is lethal to cells in the absence of P1 $c1^+$ repressor. Only about 30bp downstream of the *kilA* gene is another open reading frame, whose product of about 281 codons, designated RepL, is indispensable for *oriL* functioning. The L lytic replicon is believed to be located within a 1092bp region spanning 205bp of the 3'-end of the *kilA* gene and the entire *repL* gene, since the 1.1kb region is the only region common to all L replicon-driven plasmids (Hansen, 1989). Both KilA protein and RepL protein may be made from the same transcript, as they are transcribed from the same *c*1-regulated promoter. However, the *kilA* gene is neither essential to the activity of the L replicon, because all isolated plasmid replicons are $kilA^-$, nor to the lytic development of phages, since deletion of the region containing the kilA::IS50 insertion could restore the plaque-forming ability of P1 phages and allows phages to give clear plaques. Therefore, blockage of the activity of the L-replicon is not due to inactivation of KilA protein, but rather due to a polar effect on the transcript from the *c*1-regulated promoter (Hansen, 1989). So far, the

functions of *kilA* are not yet clear, but it is most likely that *kilA* plays a role in control of the L-replicon. Immediately upstream of kilA is the *ant* gene, consisting of two open reading frames, named as *ant1* and *ant2* (Fig. 2). In accordance with the size of their proteins, two proteins of 27kD and 40kD, RebA and RebB (Heilmann *et al.*, 1980) are assigned to these two genes. They antagonise the *c*1 repressor and appear to be made from the same transcript as is *c*4 repressor and are under the control of *c*4 repressor. Along with *kilA*, the *ant* gene products seem to be involved in lysis/lysogeny decision, though they are not essential to the functioning of lytic replication either. However, the ant gene functions are not yet fully understood.

The P1dR replicon, another P1 replicon in addition to R and L, has been isolated by Froehlich *et al.* (1986) from P1 Ap Cm (Scott *et al.*, 1982) by plating cells harbouring the low copy number plasmid P1 Ap Cm on high concentrations of both Ampicillin and Chloramphenicol. The P1dR plasmid, about half the size of P1 Ap Cm, is an independent replicon, though it is missing both the R and L replicons, since it could be stably transformed into a strain containing no P1 prophage. It appears to require rec^+ for plasmid maintenance (Froehlich *et al.*, 1986). Therefore, the P1dR replicon is supposed to be likely to be involved in late lytic replication, processing the transition from θ replication mode to σ replication mode (see above, Cohen, 1983). However, further investigation by the Scott group on this hypothesis has not been published.

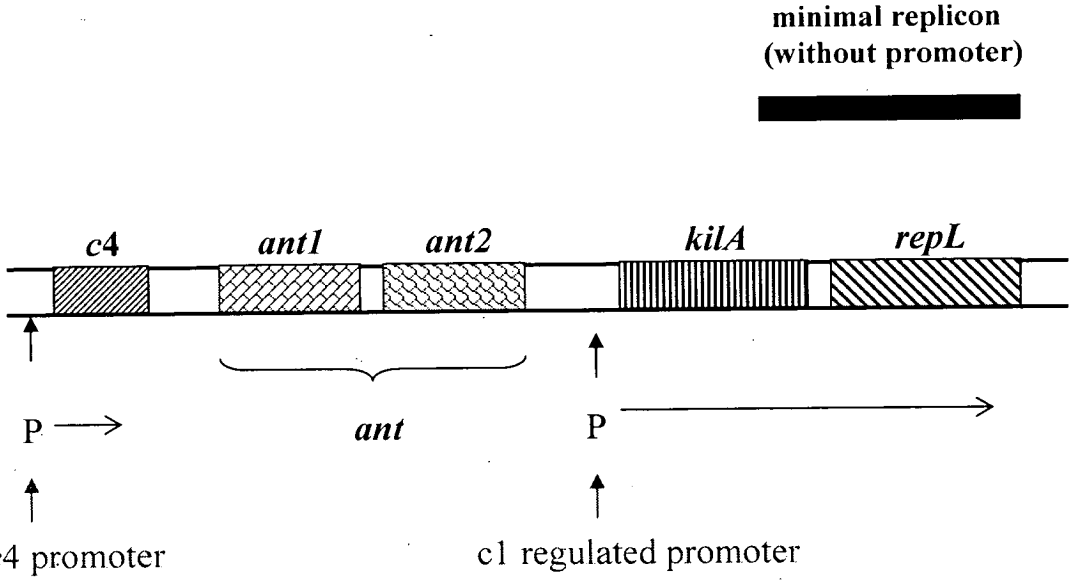


Fig 2. Elements present in the region around the L replicon. (modified from Hansen, 1989).

To carry out P1 vegetative replication and lytic development properly, some host functions are also required. It has been long known that P1 encodes a *dnaB* analogue that can complement a *dnaB* defect and allow P1 to replicate in a *dnaB*ts mutant strain at nonpermissive temperature (Ogawa, 1975; Lanka and Schuster, 1970). Although no *dnaA* analogue encoded by P1 is found, P1 can somehow still carry out lytic replication in a *dnaA* null mutant, suggesting that *dnaA* functions are not essential to P1 vegetative replication (Hay and Cohen, 1983; Hansen and Yarmolinsky, 1986). In contrast to *dnaA* and *dnaB* functions, the bacterial *dnaC* function seems to be essential to P1 vegetative replication (Nainen, 1975; Hay and Cohen, 1983). In addition, bacterial genes involved in DNA elongation, such as *dnaE*, *dnaZ* and *danG* are also required for lytic replication (Nainen, 1975; Hay and Cohen, 1983).

Studies showed that the gene expression strategy of phage P1 appears to be quite different from that of other phages like λ and P2. Most P1 genes are transcribed in lysogens, suggesting that the repression of the vegetative promoter is not complete (Mural, 1978; Sternberg and Hoess, 1983), whereas for phage λ and P2, only very little of the prophage genome is transcribed. According to the timing and the functions that P1 requires in a phage life cycle, P1 genes usually are divided into two classes. One is the early genes, consisting of genes involved in DNA synthesis, recombination and regulation of late gene transcription. The other is the late genes, which are the genes involved in phage morphogenesis and cell lysis. It seems that replicative mechanisms alone can regulate the expression of early vegetative genes (for review, Yarmolinsky and Sternberg, 1988), since removal of the activity of the L-replicon could allow induced lysogens to survive (see above).

Conversion from the lysogenic to the lytic state is triggered by increasing the lytic gene dosage due to vegetative replication as well as regulation of the expression of vegetative genes. Many studies have been carried out to investigate regulation of expression of early and late vegetative genes at the transcriptional level. One well-known P1 early gene, gene 10, and its product have been long known to be essential for regulating the production of the late viral proteins (Walker and Walker 1980, 1983). More recent work carried out by Lehnherr *et al.* (1991) showed that P1 gene 10 encodes an 18.1kd protein, gp10 (also called Lpa – late promoter activator), acting as a trans-activating factor required for late gene expression. Deletion and sequence analysis revealed that a potential promoter upstream of gene 10, designated Pr54, is found. This promoter has a typical *E. coli* consensus promoter sequence. At a region overlapping the -35 region of Pr54, a consensus-like c1 repressor-binding site

with only 3 mismatches (underlined), ATTGCTCTAATGTATTG, is found and named Op94, suggesting transcription from Pr94 is under the regulation of C1 repressor (Lehnherr *et al.*, 1992a). A DNA mobility shift assay showed that C1 binds directly and specifically to Op94, but the affinity is not as strong as that of C1 repressor to Op72. This is consistent with the observation that C1 repressor alone cannot fully repress the expression of gene 10 in vivo. However, in the presence of the product of the *bof* gene, the expression of gene 10 can be completely repressed by C1 repressor protein, although Bof itself is not able to bind to DNA. This observation is in accordance with the DNA retardation results where Bof protein significantly facilitates the affinity of C1 proteins to Op94 (Lehnherr *et al.*, 1992a). It was also observed that the expression of gene 10 starts 10-15min after the onset of lytic growth and no expression can be detected during the lysogenic state (Lehnherr *et al.*, 1992a), whereas the products of the late genes can be detected about 20 to 30min after the onset of lytic growth (Guidolin *et al.*, 1989; Lehnherr *et al.*, 1992b). The finding that the expression of gene 10 occurs at about the same time as the expression of the adjacent gene 9 (pac*A*) is consistent with the idea that genes 10 and 9 are on the same operon transcribed from Pr94/Op94 (Lehnherr *et al.*, 1991; Skorupski *et al.*, 1992).

As one of the gp10 target promoters (Walker and Walker 1980, 1983; Lehnherr *et al.*, 1991; Hansen *et al.*, 2003), the late gene promoter, Ps, which directs the expression of the tail fibre genes, has been much studied by Lehnherr *et al.* (1992b), using mutation strategies and comparison to the features of well-known promoters. It has been known for a long time that P1 requires host RNA polymerase throughout lytic development. Lehnherr *et al.* (1992b) found that Ps has a bipartite structure. First, it contains a nearly perfect -10 region. By comparison to the P2/P4 system where the host RNA polymerase acts at the conserved -10 region along with σ^{70}, Lehnherr *et al.* (1992b) proposed that P1 may also require the host σ^{70} holoenzyme to carry out transcription from its late gene promoters, such as Ps. Second, no apparent -35 region was found in Ps, but a putative operator region, since mutations within this region significantly reduce the promoter activities, called the late operator, is located around the -22 region with a dyad symmetric sequence, AAGTTACTT. In addition, the spacing between the -10 and the -22 sites appears to be rather important as well, since insertions in this region significantly reduce promoter activity. Comparison to the CRP-cAMP promoter-activating system in the *melR* promoter (Gaston *et al.*, 1990) with the above findings led Lehnherr *et al.* to propose that the late operator might be the site where a gp10 dimer binds.

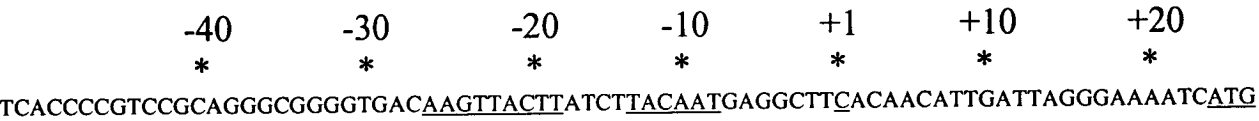


Fig 3 Nucleotide sequence of the P1 late promoter P_s

Further studies showed that to activate transcription from Ps, gp10 alone is not enough and at least one host encoded protein, SspA (Stringent starvation protein A), plays a very important role in the regulation of this process (Williams *et al.*, 1991; Hansen *et al.*, 2003). In the absence of SspA, gp10 expressed either from a prophage or from a plasmid induced by IPTG failed to activate the expression of *lacZ* from the Ps promoter after prophage induction (Hansen *et al.*, 2003). This result is consistent with the results from transcription assay, where transcription from the Ps promoter can only occur when both gp10 and SspA are present together, whereas transcription from a normal $E\sigma^{70}$ promoter appears gp10- and SspA-independent (Hansen *et al.*, 2003). Furthermore, the possibility that gp10/SspA acts as a sigma factor which associates with the core RNA polymerase (RNAP) to initiate transcription from Ps is ruled out by this assay, since in the presence of gp10 and SspA, only the holoenzyme containing σ^{70} can initiate transcription from the Ps promoter (Hansen *et al.*, 2003). DNA mobility shift assay and DNase I footprinting on promoter Ps give further support to the above observation. In the assay, both gp10 and SspA are required to stabilize the binding of RNAP to a fragment containing the late promoter Ps. gp10 binding to the -22 region of promoter Ps and its enhancement by SspA was confirmed by footprinting, although SspA itself is not able to bind to this promoter sequence. Moreover, SspA appears to be able to downregulate the expression of early genes during lytic growth, since the expression directed from an early gene promoter persists in the SspA mutant background, whereas it is downregulated in the wild type background (Williams *et al.*, 1991; Hansen *et al.*, 2003). The above results account for the observation by Williams *et al.* (1991) that a Δ*sspA* mutant does not affect lysogenic growth and does not inhibit P1 DNA replication but affects P1 development following P1 DNA replication. This suggests that along with gp10, the host SspA protein plays a role in the switch between P1 early and late transcription.

Methylation may also play a role in regulation of vegetative gene expression on the transcriptional level. This is based on the observation of regulation of *cre* expression. One of the *cre* promoters, Pcre1, contains 2 Dam methylation sites in its -35 region, and transcription from Pcre1 is sensitive to methylation by the host Dam protein. In fact, the activity of the Pcre1 promoter is 5-fold higher in a Dam^- strain than in a Dam^+ strain (Sternberg *et al.*, 1986). This suggests that the Dam methylation system might be used by P1 to shut off genes that are no longer needed for phage growth.

After P1 morphogenesis-genes are expressed, phage capsids are assembled, and the P1 concatemeric DNA is ready, the phage DNA will then be packaged into the phage heads using a headful mechanism (for details, see below).

Now it is time for P1 to get free. For most bacteriophages, lysis of the host usually requires at least two proteins, holin and endolysin. Holins are small bacteriophage-encoded membrane proteins, which create holes on the membrane. Endolysin is a term for muralytic enzymes that degrade the cell wall, such as the product of the T4 e gene, a lysozyme and the λ R protein, a transglycosylase. Endolysins require the assistance of membrane-disrupting proteins in order to reach the peptidoglycan of the cell wall (Wang, *et al.*, 2000; Schmidt *et al.*, 1996). Evidence shows that holins accumulate and oligomerize in the membrane throughout the period of late-gene expression, suggesting a timing mechanism in which holin accumulation causes a proton (or other ion) leak and gradually titrates the protein-motive force (pmf) until suddenly triggering a lesion that permeablizes the membrane (Fig 3). Therefore, it was believed that holin is the membrane-disrupting protein that controls the functional access of the endolysin to the murein by permeabilizing the membrane and in consequence, the length of the vegetative cycle (Grundling *et al.*, 2001; Wang *et al.*, 2000).

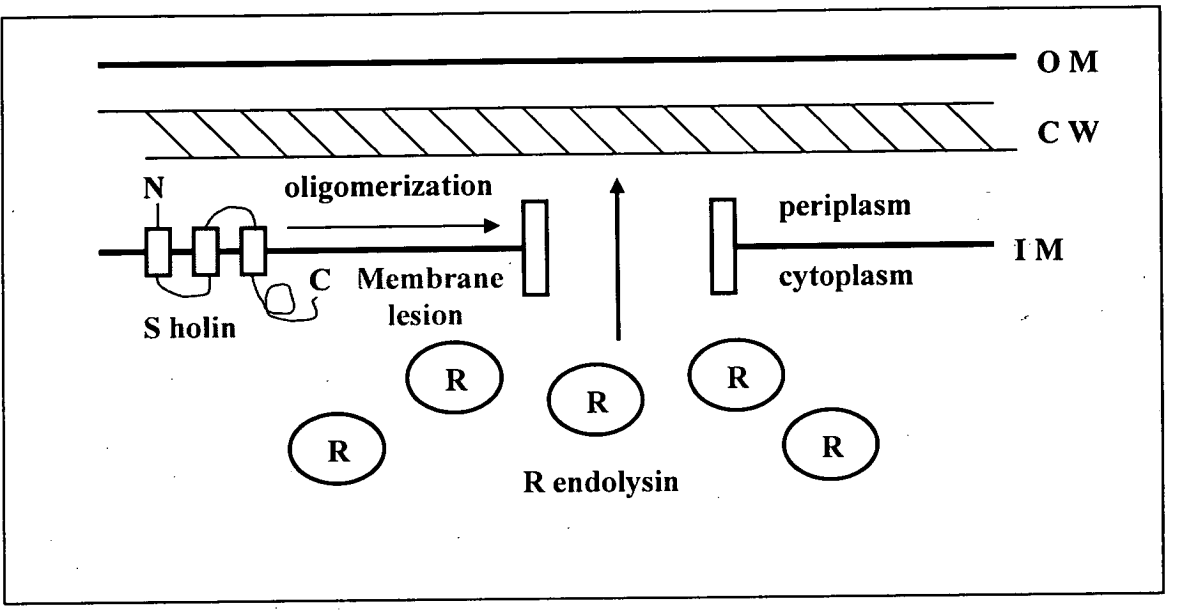


Fig 4. The holin-endolysin system of bacteriophage λ. The details are described in the text. OM: outer membrane; CW: cell wall; IM: inner membrane. (modified from Bernhardt *et al.* 2002).

Three genes in the P1 genome related to cell lysis, *lydA*, *lydB* and gene 17 have been found by mutational approach. *lydA* and *lydB* are in the same operon (*dar*) and are under the control of a gp10-regulated promoter P*dar*. A mutant lacking both *lydA* and *lydB*, P1Cm2, exhibited a delayed and gradual lysis phenotype and formed tiny plaques on a bacterial lawn. Infected cells that have not lysed can be lysed with chloroform and continue to produce phage beyond the normal lysis time. Gene 17 is located just counterclockwise from the IS1 element and is under the control of the C1 repressor-controlled promoter Pr21. A mutant carrying an amber mutation in gene 17 is completely defective for cell lysis, but the lytic development of the phages is unaffected and assembly of the phage particles stops at the normal time of cell lysis (Schmidt *et al.*, 1996; Yarmolinsky and Sternberg, review, 1988). In addition, the cells are not lysed by chloroform treatment (Walker and Walker, 1980).

The above results indicate that *LydA* and *LydB* might be holins and gp 17 probably a lysozyme. Sequence analysis revealed that the secondary motif of *LydA* protein contains a holin protein-like structure. However, no homology of *LydB* to other phage lysis proteins was found. A mutant, am2.3, having an amber mutation in the *lydB* gene was found to prematurely lyse its host cell. This

observation along with expression results, where cells harbouring a *lydB*-deleted plasmid were found to be lysed much more quickly after induction relative to cells bearing a plasmid containing both *lydA* and *lydB*, suggest that *LydB* acts as an antagonist of *LydA* to prevent premature cell lysis (Schmidt *et al.*, 1996). This feature is similar to the λ lysis strategy. There are also three proteins involved in cell lysis for λ. R protein is an endolysin. The *S* gene produces two proteins – the holin and the holin inhibitor, designated S105 and S107, from the same gene. The difference between these two proteins is an extra positively charged residue (Lys_2) in S107. This is because the translational initiation of S105 is at codon 3, whereas the translational initiation of S107 is at codon 1. Premature cell lysis occurs when only S105 is expressed in the infected cell, while S107 is found to prevent the premature lysis by S105 (Wang *et al.*, 2000). By analogy to the phage λ holins, *LydA* exerts the function of S105 and *LydB* exerts the one of S107. Alignment of the amino acid sequences of gp 17 to other phage lysis proteins also revealed homology to the T4 lysozyme family. Therefore, it is possible that *LydA* and *LydB* are working together to ensure that cell lysis occurs at the right time, while gp 17 finishes the final step – breaking the cell wall to allow phages to be released.

Another important and interesting feature of P1 is how P1 protects its DNA after injection of its DNA into the host cell, since its DNA is linear in the capsid and remains so before cyclization by the recombination system in the new host. Iida *et al.*, (1987) found that P1 is only weakly cut when it infects cells carrying the type I restriction and modification system, whereas P1 DNA purified from P1 heads is easily digested in vitro, suggesting that P1 encodes a system antagonistic to the type I restriction enzymes.

Two phage genes, *darA* and *darB*, have been found to be involved in preventing restriction. *darA* is located in the *dar* operon, the same operon which contains the *lyd* genes, and is also under the control of the gp10-regulated promoter P*dar* (Iida *et al.*, 1987; Iida *et al.*, 1998). *darA* encodes a 68kD protein, which along with DarB protein, appears to be associated with the P1 head, since they both are found in purified heads isolated from a lysate of P1Cm0 *sus*50 (Yamamoto, 1982). *darB* is located near coordinate 15 of the P1 map and encodes a rather big protein, about 200kD.

The type I restriction enzymes are 900kD ATP-dependent complexes, and have both methylation and restriction activities. Type I enzymes recognize specific sequences but cut the DNA at remote sites

that can be thousands of base pairs away from the recognition site (Horuchi and Zinder, 1972). DarA protein protects P1 DNA from cleavage by the *Eco*A family enzymes, whereas DarB is thought to protect against the *Eco*K family enzymes in conjunction with DarA, since *darA* mutants lose both DarA and DarB functions and therefore are sensitive to both *Eco*A and *Eco*K family enzymes. Mutants carrying a *darB* mutation will only lose the DarB functions; the DarA functions such as *Eco*A resistance remain (Iida *et al.*, 1987; Yarmolinsky and Sternberg, review, 1988). Iida and his collaborators (1987) demonstrated that the Dar proteins do not protect DNA against restriction in the cells where they are synthesized, but rather follow the DNA to be injected into the recipient cell and then protect the DNA from restriction in the recipient. In fact, it appears that any DNA, including transducing DNA, packaged into a wild-type P1 virion will be protected from restriction. However, the Dar proteins do not function in *trans* to protect the DNA injected by other phages. More information about Dar proteins will be discussed later.

1.2. DNA packaging and cleavage at the P1 *pac* site

1.2.1. "headful"packaging

P1 DNA is unidirectionally packaged into the P1 head by the headful (Fig 4). Before packaging, P1 genomes are in the form of a long concatemer consisting of repeating monomer units of viral DNA arranged in head-to-tail configuration. P1 packaging enzyme, called Pacase, then recognizes a specific site, called the *pac* site, and cleaves it to generate two ends. The end destined to be packaged will move into the P1 capsid until the capsid is full, meanwhile the upstream end is rapidly degraded by host nucleases. A second cut at a non-specific site one headful distant from the *pac* site separates the packaged P1 DNA from the rest of the concatemer. A second round of the packaging is reinitiated from the end generated by the headful cut. This process is called headful packaging and produces DNA that is terminally redundant and cyclically permuted.

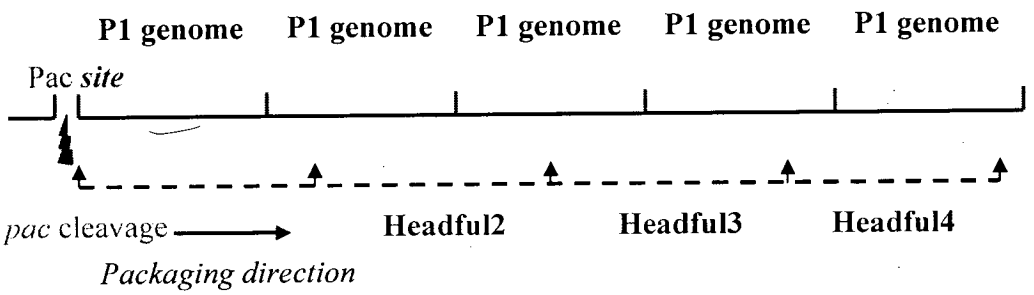


Fig 5. Cartoon showing the headful packaging mechanism

1.2.2. The *pac* site

As with other circularly permuted bacteriophages, such as P22, T4 and so on, the packaging of bacteriophage P1 DNA starts off at a specific site, called the *pac* site. *pac* is located on the *EcoR*I-22 proximal side of P1 *EcoR*I fragment 20. A full functional *pac* site is a 161bp DNA sequence, consisting of 4 hexanucleotide "TGATCA" repeats at one end and 3 at the other, flanking a 13bp sequence in which cleavage by Pacase occurs. Cleavage by Pacase generates two termini with 2bp 3'-protruding single stranded ends (Sternberg & Coulby, 1987b) (Fig 5). The GATC [within TGATCA elements] is the Dam methylation site, and it has been reported that methylation on the *pac* site is essential for recognition and cleavage of *pac* sites by Pacases (Sternberg and Coulby, 1990). Moreover, it seems that the functions of these hexanucleotide repeats are additive, since deletion assay showed that the activity of a mutant *pac* site is proportional to the number of these repeasts remaining (Sternberg and Coulby, 1987b). Sternberg and Coulby (1987a) also demonstrated that the *pac* end not destined to be packaged is degraded by at least 2 host nucleases, one of which must be RecBCD, also called Exonuclease V. This is based on the following observations. First, the *pac* end destined to be packaged into a P1 head was detected 5 to 20 times more efficiently than was the other end of *pac*. In addition, after P1 infection, neither end of a *pac* site on a small plasmid could be detected efficiently in a *recBCD*$^+$ strain, whereas in a *recBCD* strain, cleavage pattern of the plasmid *pac* substrate resulted in a similar pattern generated by cleavage at a chromosomally integrated *pac* substrate. This suggests that RecBCD degraded the linear DNA from the unprotected end of two *pac* ends generated by *pac* cleavage and marched down the DNA. The chromosomal DNA is so long that the enzyme was not able to progress to the other end, whereas the plasmid size is so small that RecBCD was able to digest the entire molecule within a short time.

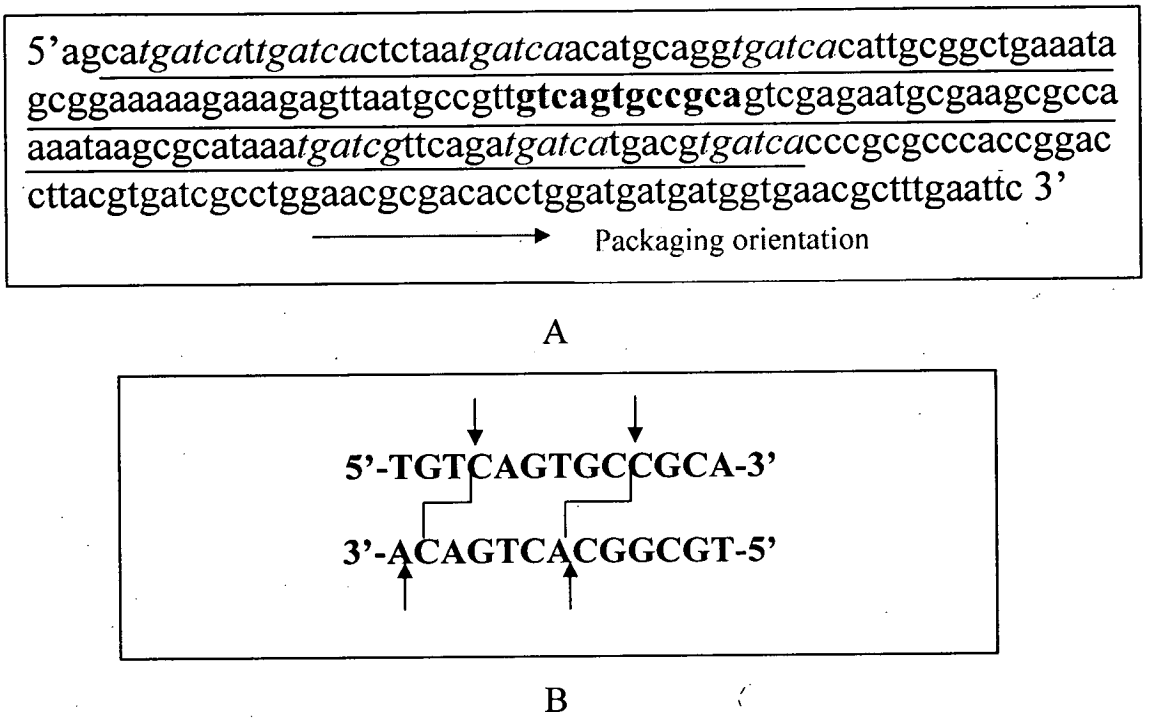


Fig 6. Sequence of the *pac* site and the cleavage site by the Pacase. **A)** the sequence of the *pac* site. The hexanucleotides are in italic and the cleavage site is in capital. **B)** The cleavage site. Arrows show the cutting points by the Pacase, producing 2bp 3'-protruding overhangs. The *pac* site is underlined. (from Sternberg and Coulby, 1987b)

1.2.3. Pacases

Pacases of the double stranded DNA bacteriophages, such as λ, T7, T4 and P22, are all usually composed of two subunits, one large and one small, encoded by distinct and usually adjacent genes (Casjens, 1985; Black, 1989). Two proteins involved in P1 DNA packaging have been located and identified. One is PacA, 45 kDa and the other is PacB, 56 kDa (Skorupski *et al.*, 1992). They are expressed from a C1 repressor-regulated promoter that lies immediately upstream of gene10, thus appearing to be co-ordinately expressed with gene10 (Lehnherr *et al.*, 1991; Skorupski *et al.*, 1992)

(Fig 7), whose product is essential for the synthesis of most "late" P1 proteins (Walker & Walker, 1983, see above). The pac*A* gene (originally called gene 9) is located 85bp downstream of gene 10 and starts from a GTG codon. The *pac* site is located within the pac*A* gene at the gene 10-proximal end. The start codon, ATG, of the pac*B* gene overlaps with the last "A" of the stop codon of pac*A* by 1 base pair. A putative open reading frame, designated pac*C*, is found within the 3'-end of pac*B* gene (Skorupski *et al.*, 1992). Skorupski *et al.* (1992) also found that the efficiency of expression of PacA and PacB is very low even when a very strong promoter, the T7 promoter, is used for expression. The expressed proteins can only be detected by labelling with S^{35}. Furthermore, expressing both PacA and PacB simultaneously resulted in a lower yield of both proteins relative to expression of either PacA protein or PacB protein alone. Also expression was diminished following an extended induction time. Possibly, the plasmid expressing the proteins becomes a target attacked by these two proteins, since the pac*A* gene contains the *pac* site. The low level expression of Pacase is a common feature for many phages (Murialdo *et al.*, 1987; Rao and Black, 1988). This is possibly due to the weak ribosome-binding sites of the Pacase genes, since replacement of the original phage RBS with a more efficient RBS resulting in a significant improvement was observed in both P1 and λ (Skorupski *et al.*, 1992; Murialdo *et al.*, 1987).

Expression of the Pacase is at an early stage of the P1 growth cycle, because *pac* cleavage can be detected within 10 to 15 min after P1 infection (Sternberg & Coulby, 1987a), suggesting that the P1 Pacase may not only play a role in cleavage of the *pac* site, but also another role in the P1 life cycle. Skorupski (1994a) demonstrated by gel retardation experiments that PacA can recognise and bind to methylated *pac* DNA independently of PacB, but requires an *E. coli* extract containing the PacB protein for DNA cleavage, suggesting that PacA is the binding subunit of the P1 Pacase responsible for DNA recognition. This conclusion is further supported by DNaseI footprinting, in which PacA appears to bind to the hexanucleotide repeats in *pac* (Skorupski *et al.*, 1994b). It also appears that the binding of PacA to DNA is under the regulation of host factors, since purified PacA can only bind to the *pac* site in the presence of the wild type *E. coli* extract, otherwise neither purified proteins nor the cellular extract alone can form DNA-protein complexes (Skorupski *et al.*, 1994a). Two host factors, IHF and HU, were found to contribute to *pac* cleavage (Skorupski *et al.*, 1994a, b). IHF, integration host factor, showed binding ability to the consensus IHF binding sequence in the *pac* site and the ability to facilitate the independent binding of PacA to each hexanucleotide domain in DNaseI

cleavage and gel retardation experiments. In addition to IHF, HU appeared to affect the fidelity and efficiency of *pac* cleavage in nuclease activity assays in vitro. Based on the above observations, a model for *pac* cleavage has been made by Skorupski (1994b) (Fig 8)

Interestingly, studies also showed that an 80 to 90-fold molar excess of PacA relative to *pac* is required for normal *pac* cleavage. This could possibly be explained by the fact that there are multiple PacA binding sites (the hexanucleotide repeats), up to seven, in a single functional *pac* site, and a PacA complex, such as a PacA tetramer, is required to function as a binding subunit of a pacase (Skorupski *et al.*, 1994a). The mechanism of PacB action is not yet very clear, but most likely it interacts with ATP since an ATP-binding consensus sequence with pac*B* was found.

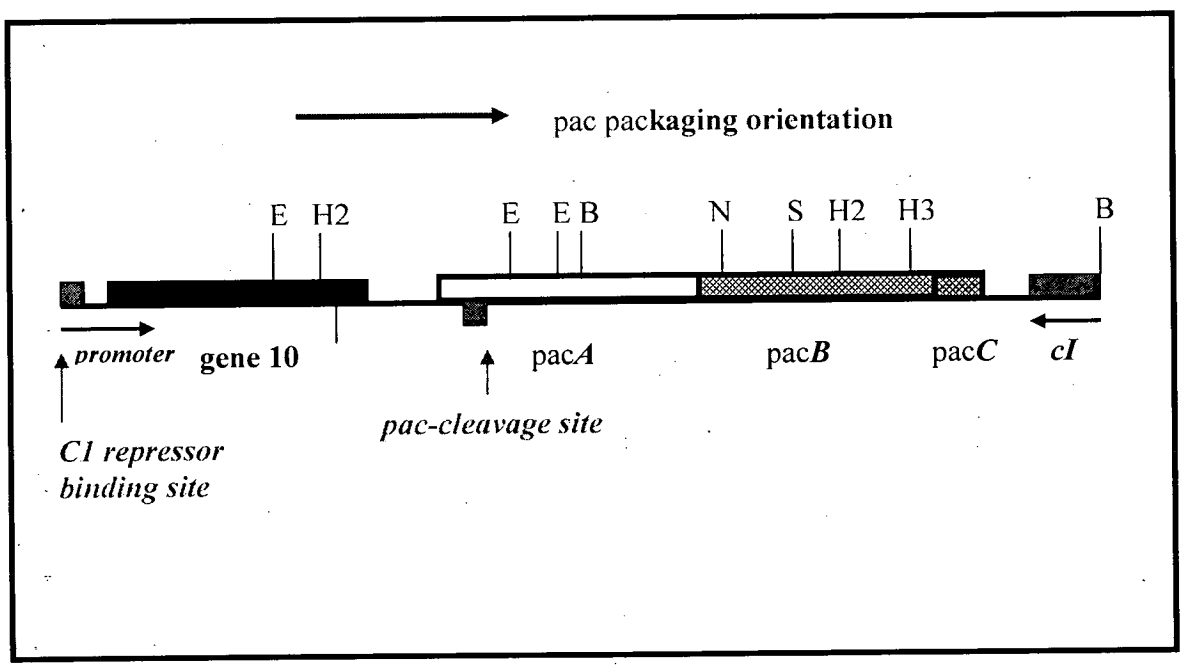


Fig 7 A map of approximately 4kb from the *pac* region of P1 DNA. The detailed description is in the text. E, EcoRI; H2, HincII; B, BglII; N, NotI; H3, HaeIII; S, SmaI. (modified from Skorupski *et al.*, 1992)

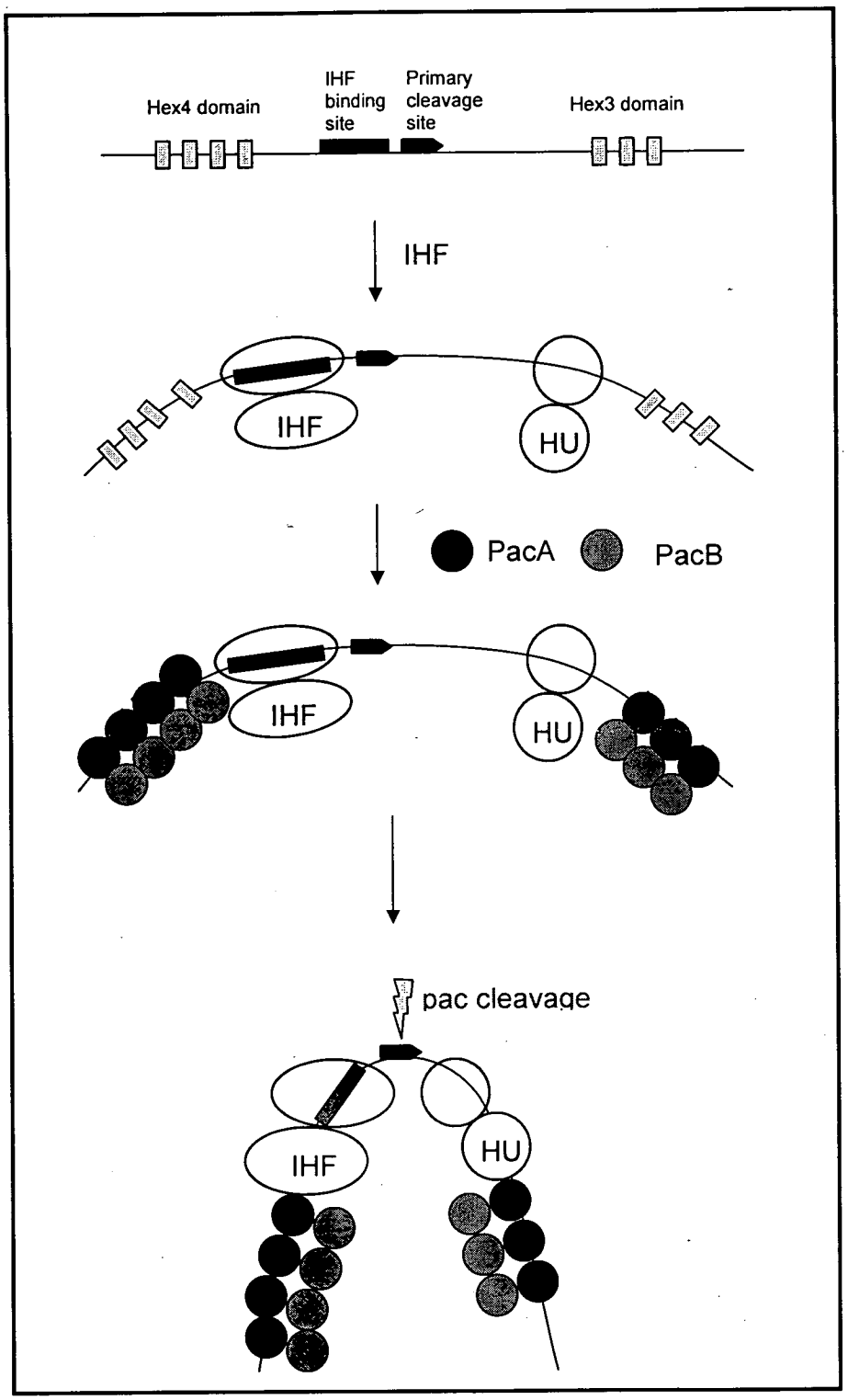


Fig 8. A model for *pac* cleavage (modified from Skorupski *et al.*, 1994b)

1.2.4. Regulation of packaging

Although the packaging of viral DNA needs a phage head to be available for packaging to begin, the Pacase appears to be expressed early during the phage growth cycle. The early expression of the Pacase genes suggests that P1 Pacases may play other roles in the P1 life cycle in addition to packaging. On the other hand, the early expression of the Pacase genes also causes a problem: how is *pac* cleavage avoided during the early stage of phage infection as both the *pac* site and enzyme are present in the cells? In addition, P1 packages its DNA by a processive headful packaging and generates terminal redundancy, which is critical for the cyclization of the viral DNA after infection. Therefore, it is important to prevent the cleavage of P1 concatemer at each *pac* site. To ensure that only the initial *pac* site is the cleavage target but not the others, the regulation of cutting by pacases is indispensable. It is known that the methylation of at least some of the hexanucleotide elements in the *pac* site by Dam methylase is essential for cleavage (Sternberg & Coulby, 1990). It has also been observed that hemimethylated DNA appears to be substrate for Pacase binding but not for *pac* cleavage (N.Sternberg, unpublished). Also, it was observed that the host DNA in a dam⁻ mutant was largely methylated 20min after P1 infection, suggesting that P1 encodes its own methylase, which is expressed about halfway through the P1 life cycle (Sternberg & Coulby, 1990). The above phenomena give rise to a model which could possibly account for the regulation of *pac* cleavage: the *pac* site is transiently hemimethylated by the host Dam methylase as it is replicated onto the tail of the rolling circle. Then the PacA protein recognizes and binds to *pac* and prevents it from becoming fully methylated by the bacterial Dam methylase, since PacA proteins possibly bind to the hemimethylated *pac* more avidly than the host Dam methylase does and remain so during the early stage of infection. Late after infection, methylases produced by P1 itself should become very abundant in the cells for there are many copies of the P1 methylase gene so that methylases in the cells could compete with PacA protein to bind to the *pac* site and therefore, the *pac* site will be methylated and activated to become a target for the P1 pacases at the right time (Yarmolinsky and Sternberg, review 1988; Skorupski *et al.* 1994a).

1.3. Transduction and abortive transduction

1.3.1. Introduction

Transfer of genetic material between bacterial cells is a common event in both laboratory and the wild environment. Mainly, three processes allow genetic material to be transferred from a donor cell to a recipient cell, termed transformation, conjugation and transduction. Transformation is the direct transfer of naked DNA into the recipient, while DNA transfer between cells by conjugation requires cell-cell contact via structures encoded principally by plasmids. Transduction is the transfer from a donor cell to a recipient cell of nonviral DNA by bacteriophages; transduced DNA is protected in a viral coat (Masters, 2003).

Specialized and generalized transductions are two types of transduction processes with easily distinguishable features (Table 1). As a representative, Phage λ is a well-studied phage for carrying out specialized transduction. In the lysogenic cycle, Phage λ is integrated into the host chromosome via a specific recombination event between a site, attB, on the chromosome and a site, attP, on the phage. Specialized transducing particles are formed when excision occurs inaccurately to form a circle consisting of both part of the phage genome and a part of the host chromosome which is next to the integration site. Once the specialized transducing particle infects a recipient cell, the transducing DNA injected into the cell by phage λ can either be integrated into the recipient chromosome by a single crossover at a homologous site to form an unstable lysogen or normal lysogeny can occur to form a stable lysogen. In contrast to specialized transduction in which only the chromosomal markers next to the phage integration can be transduced, generalized transducing particles can be formed either by induction of a lysogen or by infection and can transfer any chromosomal markers to the recipient. Because the mechanism of formation of generalized transducing DNA is different from that of specialized transducing DNA in λ, phage P1 contains only linear host DNA in its capsid. After infection, since the phage DNA is linear, the transduced DNA is integrated into the chromosome via a

double crossover event between the homologous region on the recipient chromosome and the transduced DNA to form a stable haploid recombination.

Table 1 Comparison of specialized and generalized transduction (from Masters, 2003)

	Specialized	Generalized
Genes transduced	Adjacent to chromosomal insertion site	Any gene
Process creating transducing particles	Induction of lysogen	Induction or infection
Transducing particles	Contain both phage and host DNA covalently linked on a single molecule	Host DNA only
Process creating transducing particles	Aberrant excision of lysogenic phage DNA	Mischoice of packaging substrate
Transduced progeny	Unstable, partially diploid lysogens	Stable, haploid recombinants
Best-known enterobacterial transducing phages	λ and related phages	P1, P22 and T4

1.3.2. Events in the donor cell

As mentioned above, for reasons unknown, occassionally the P1 packaging machinery, instead of packaging the P1 genome, treats the host chromosomal DNA as a packaging substrate and packages it into P1 heads, giving rise to a small proportion of so-called transducing particles in every lysate. Only about 0.3% of total phages are transducing particles in every P1kc lysate, which is the most common phage P1 strain used in the laboratory (Ikeda and Tomizawa, 1965a, b; Sandri and Berger, 1980a), while in a lysate of P1607H, a high-frequency transducing mutant, transducing particles represent about 2% of the total phage yield (Sandri and Berger, 1980a; Wall and Harriman, 1974). Although the source of the phage used in my work is not clear now, about 5% of total phages are

transducing particles in the lysates made by this phage, according to calculation done by M. Hanks before. As described above, the DNA in the heads of transducing particles is the host DNA only.

How is phage P1 able to package the chromosomal DNA instead of the P1 genome? The simplest explanation is that there are *pac* sites or *pac*-like sites on the chromosome. For *Salmonella* phage P22, packaging of the host DNA is highly likely to be from *pac*-like sites, since the transduction frequencies of markers on the chromosome by P22 are quite different, varying over a range of 1000-fold, suggesting that there are some hot-spots of cleavage for P22 Pacase (Masters, 2003). However, this seems not to be the case for phage P1.

Sternberg and Coulby (1987a) made a λ lysogen with a λ vector, on which a P1 *Eco*RI-20 fragment, in which the *pac* site is located, is cloned. The lysogen was infected by P1 and the resulting lysate was tested for the transduction frequencies of the markers on the chromosome. Up to an 80-fold increase of the transduction frequencies of the chromosomal markers to one side of the *Eco*RI-20 fragment was observed relative to a non-lysogenic control. Hanks (1988) demonstrated that there are no P1 *pac* sites on the chromosome and showed by quantitative Southern hybridisations that most markers are packaged at similar levels, varying over a quite small range, about 3-fold. These observations suggest that initiation of packaging of the chromosome by P1 is probably from many sites all over the chromosome, rather than from *pac*-like sites (Hanks, M.C. *et al.*, 1988). However, the actual mechanism of the initiation of packaging of the chromosomal DNA by P1 is still unclear.

1.3.3. Events in the recipient cell

1.3.3.1. Transduction frequency

Although the above observations showed that all the markers on the chromosome are packaged by P1 at similar levels, the transduction frequencies of different markers are apparently different, varying over a range of up to 30-fold (Masters, 1977). This result suggests that the differences

amongst markers in P1 transduction frequency are due to events in the recipient cell rather than in the donor cells. Masters (1977) showed that the markers around the origin of replication of the *E. coli* chromosome are transduced highly. She found that markers near the origin are transduced with a very high frequency, resulting in a peak, while the transduction frequencies of the markers flanking this peak are much lower, resulting in troughs on either side of the peak. This finding perfectly matches the gene frequency distribution on the chromosome (Bachmann *et al.*, 1976), thus P1 transduces best regions of high gene density. Since the gene frequency probably reflects a possibility that the genes concentrated in these regions may be important in function and thus more accessible to transcription and translation than others, to explain the above finding, Masters proposed that either the DNA in these high gene frequency regions is more accessible to phage enzymes during DNA packaging than the DNA in other regions is and therefore are amenable to transduction, or these parts of the DNA are more accessible to the recombination system in the recipient cell than other parts of the DNA. The former hypothesis can be ruled out as described above. The latter may make more sense. Strong support for this hypothesis comes from the UV irradiation experiment described below.

It has been shown that the range of variation in transduction frequencies of markers on the chromosome can be reduced by either exposure of P1 lysate to a low dose of UV before infection or UV irradiating the recipient cells before transduction. After these treatments, the transduction frequencies of markers which are usually transduced poorly are significantly increased and reach a level similar to that of the markers which are transduced best, as UV has very little effect on these markers (Newman and Masters, 1980). These results strongly suggested that discrimination in transduction frequency is mainly determined by the events in the recipient cell, possibly the recombination pathways in the recipient cells. To account for the above result, Masters and her colleagues (1984) proposed that a non-sequence-specific recombination system substitutes for the sequence-specific recombination system to direct recombinational events involving damaged DNA, while the sequence-specific recombination system plays a main role in the recombination of undamaged DNA. The candidates, *recBC* product and *recF* product, for the sequence-specific and non-sequence-specific system respectively were investigated by Masters and her collaborators (Masters *et al.*, 1984), with the following observations. First, conjugational and transductional recombination in *E. coli* mainly require the *recBC* product while an alternative mechanism dependent on the *recF* product can be used in *recBC sbcB* cells (Clark, 1973). RecBC can exert *chi* (χ sequence)

dependent effects on linkage in transductional crosses (Dower and Stahl, 1981) and are responsible for most conjugational and transductional recombination in wild type cells (Mahajan and Datta, 1979; Clark, 1980), and thus, are likely to be responsible for the sequence specific recombination reacting with undamaged DNA. In contrast, RecF participates in the slow integration of single-stranded DNA fragments and is possibly involved in the recombination of damaged DNA in wild-type cells, since it was observed that increasing the levels of *RecA* protein through mutation or SOS induction can accelerate *recF* mediated recombination in *recBC* cells (Lloyd and Thomas 1983). A *recBC sbcB* mutant with a fast-growing (FG) phenotype isolated by Masters (1984) was found to be able to increase the level of RecF dependent recombination and render it unselective, although other *recBC sbcB* mutants continue to exhibit marker discrimination. The events that lead to transductional recombination in *E. coli* and the mechanism of UV stimulation of transduction frequency are still not fully understood.

1.3.3.2. Fates of transduced DNA and stable transduction

After linear P1 DNA is injected into the recipient cells, the injected DNA has three possible fates: P1 transduced DNA could be degraded by host nucleases, recombined into the host chromosome to form stable transductants, or maintained within the cytoplasm in a form refractory both to recombination and degradation. Since the transduced DNA in the last case does not replicate, this DNA is termed abortively transduced DNA or abortive DNA and the cells harbouring this DNA are called abortive transductants.

Using ^{32}P-BU-labeled transducing particles to infect [^{3}H]thymidine-labeled recipient cells followed by analysis of the transducing DNA on CsCl equilibrium density gradients, Sandri and Berger found (1980a) that only 7-15% of the ^{32}P-BU-labeled transducing DNA becomes covalently associated with the recipient DNA. Further experimental analysis of the nature of the integrated DNA showed that, at most, 9-15% of the associated ^{32}P-BU-label could be released as sheared DNA fragments larger than 2 x 10^5 daltons (about 308 bp) in length but only from Rec$^+$ recipients. This conclusion is based on the idea that shearing or sonicating the DNA reduces DNA size and thus increases the ratio of the heavy label (integrated transducing DNA) to the light label (the recipient

DNA) in a DNA fragment. This results in the separation shift of the ^{32}P-BU-labeled DNA band on the CsCl gradient from the light recipient DNA region ([^{3}H] thymidine-label region) to the heavier region (^{32}P-BU-label region). Therefore, the above finding suggests that only a maximum 15% of the transducing DNA covalently associates with the host DNA or that only about 2% of total transducing DNA represents truly recombinant DNA. After sonication, which generates DNA fragments as short as 2 x 10^5 daltons (about 308 bp), the majority, about 85%, of the ^{32}P-BU-label is covalently associated with the recipient DNA, suggesting that this portion of the label represents small transducing DNA which is degraded by the host nucleases and recycled during DNA synthesis (Sandri and Berger, 1980a).

Therefore, according to Sandri and Berger's work, about 10% of total transducing DNA is degraded by the host nucleases in the recipient cell after infection, while only about 2% of total transducing DNA is recombined into the recipient chromosome to give rise to stable transductants which can form normal colonies on the selective plates.

1.3.3.3. Abortive transduction and abortively transduced protein

The first demonstration of abortive transduction was made by Lederberg on 1956 when he transduced nonmotile *Salmonella* to motility. Transduced progeny instead of forming trails, which motile cells usually do, consist of strings of colonies of nonmotile cells, each apparently arising from a single nonmotile descendent of a motile cell (Lederberg, 1956; Masters, 2003). This interesting phenomenon can be explained if the transduced DNA is not replicated so that after division only one daughter cell inherits the transduced DNA. This daughter would be able to make flagella and swim off, while its sister cell is nonmotile and forms a colony at the point where flagellar protein is too dilute to make flagella.

As described above in Sandri and Berger's work (1980a), about 90% of total transducing DNA extracted from the recipient cell appears as nonchromosomally associated DNA at the heavier region on the CsCl equilibrium gradient (Sandri and Berger, 1980a). Further work showed that this nonassociated abortive DNA is not replicated and can be recovered even 5hr after infection,

suggesting that this nonassociated DNA represents abortively transduced DNA (Sandri and Berger, 1980b). This conclusion is consistent with the previous finding that a majority of P1 transductants are abortive (Gross and Englesberg, 1959).

Then, questions arise: How is the abortive DNA formed? How can it be refractory to both degradation and recombination and non-replicated? The simplest answer to the above questions is that abortively transduced DNA is in a circular form so that it is resistant to attack from the host nucleases, such as RecBCD. RecBCD is an ATP-dependent helicase which, by ATP hydrolysis, unwinds double-stranded DNA, making it a substrate for recombination catalyzed by the *RecA* protein. The enzyme is also a potent nuclease on linear double-stranded DNA and single-stranded DNA and also on single-stranded circular DNA in the presence of ATP and excess Mg^{2+}. However double-stranded circular DNA molecules either of the closed circular (cc) form or the open circular (oc) form (Chen *et al.*, 1997; Mukai *et al.*, 1973) are not substrates. One of the important features of this enzyme is that the exonuclease activity of RecBCD on double-stranded DNA is suppressed when the enzyme encounters a recombinational hot spot, the so-called *chi* (χ) sequence (5'-GCTGGTGG), a very common sequence on the *E. coli* chromosome (Chen *et al.*, 1997; Yu *et al.*, 1998). However, the effect of *chi* sequence on blocking the RecBCD nuclease activity is reversible by adding excess Mg^{2+}. Previous data showed that the χ-modified RecBCD enzyme becomes a non-specific dsDNA Exonuclease in the presence of 10mM Mg^{2+}, while high concentrations of ATP or the presence of *E. coli* single strand DNA binding protein increases duplex DNA unwinding relative to DNA degradation (Yu *et al.*, 1998; Muskavitch and Linn, 1982). In addition, Ca^{2+} inhibits the entire nuclease activities of the enzyme, but does not affect the DNA unwinding and ATP hydrolysis activities (Muskavitch and Linn, 1982).

Then, how is abortively transduced DNA circularized after infection? It seems that it is not possible that the transducing DNA, which is randomly packaged from the host chromosome, is circularized by homologous recombination or annealing of homologous ends. To address this problem, ^{32}P-BU-labeled abortively transduced DNA was purified from the recipient and analyzed using velocity sedimentation and electrophoresis by Sandri and Berger (1980b). Their results suggested that abortively transduced DNA is circularized by a protein associating with two ends of the transduced DNA. This conclusion was based on the following observations: first, abortively transduced DNA appears to be sedimented 1.7 to 1.8 times faster than linear P1 phage DNA in neutral sucrose gradients,

corresponding to supercoiled and relaxed circular prophage DNA but sedimented with linear P1 phage DNA after treatment with SDS, heat (70°C) or Pronase. More direct evidence came from agarose gel electrophoresis. After electrophoresis and autoradiography, the abortively transduced DNA bands were found to comigrate with circular prophage DNA in the agarose gel. Treatment of abortively transduced DNA with sodium lauryl sulphate, heat (70°C) or Pronase also converted the DNA to a form which comigrates with linear P1 DNA, suggesting that a protein is involved in the configuration of abortively transduced DNA and probably not covalently attached to the DNA, since it can be removed by SDS and heat at 70°C (Sandri and Berger, 1980b). This finding is consistent with the observation by Ikeda and Tomizawa in 1965 (Ikeda and Tomizawa, 1965b), in which they found a protein attached only to the transducing DNA of the P1B particles but not to the DNA of infective particles or to the transducing DNA of the P1S particles. The estimated molecular weight of the protein is rather big, up to 500KD. However, the protein found by Ikeda and Tomizawa appeared to be covalently associated with the transducing DNA, since it appears stable to heating at 93°C in 1% formaldehyde for 10min, which is inconsistent with Sandri and Berger's observation.

The nature of the protein is still unclear. This is because the total amount of the protein is only about 0.8% of the amount of the transducing DNA. Considering that the transducing particles are only a very small portion of a P1 lysate, the protein, the so-called abortively transduced protein, is too rare to be easily isolated and identified (see the calculation below) and therefore it is still not known whether it is of host or viral origin (Ikeda and Tomizawa, 1965b; Sandri and Berger, 1980b; Hanks, thesis 1986).

Conditions:

Culture vol.: 500ml; cell No.: 2×10^8/ml; m.o.i. = 4

Total Input P1: $500 \times 2 \times 10^8 \times 4 = 4 \times 10^{11}$

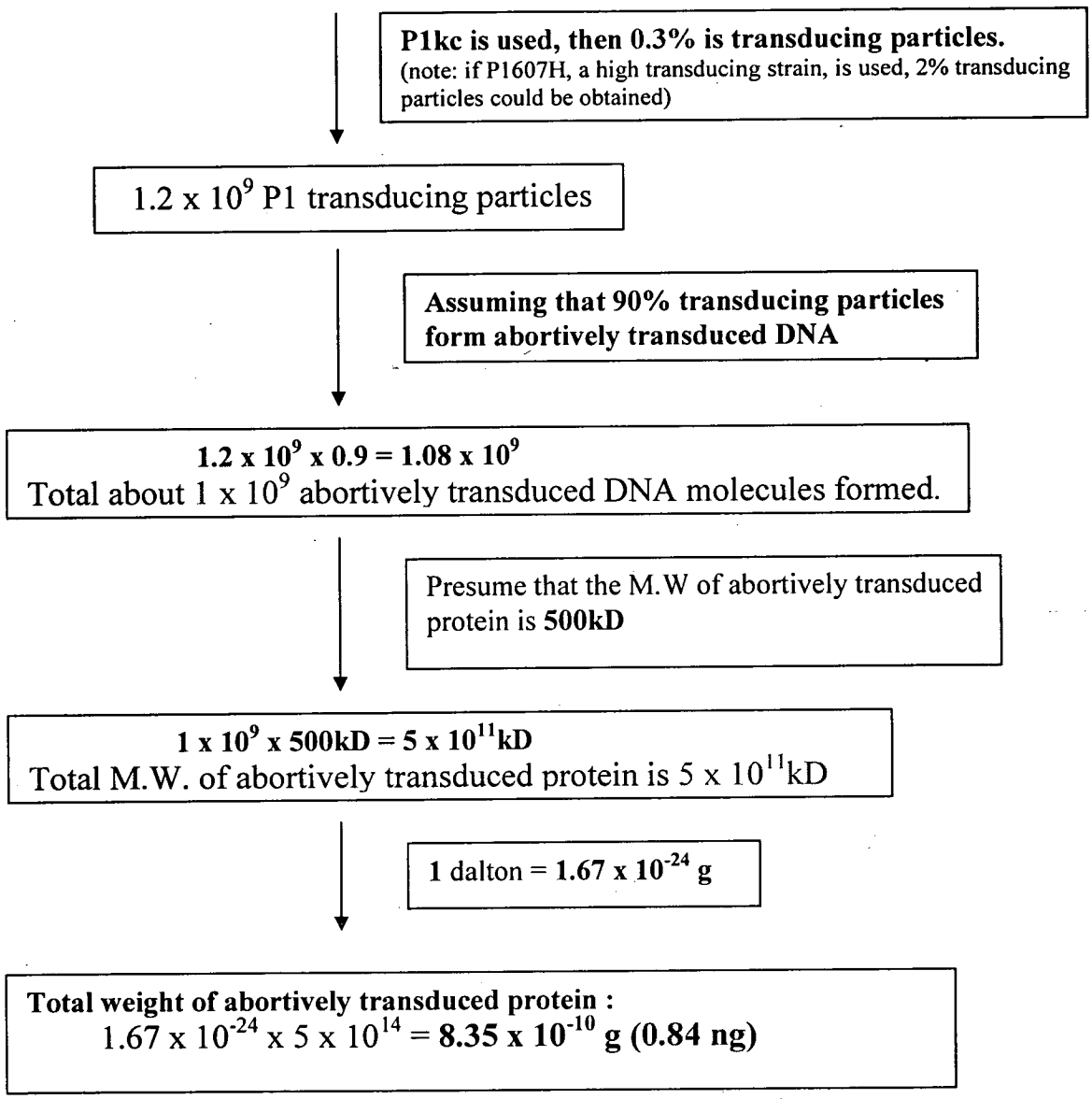


Fig 9. Calculation of the yield of the abortively transduced protein. A putative yield of AT protein from 500ml culture with a multiplicity of infection of 4 was calculated using the data from previous work.

Several hypotheses (Table 2) have been made as to the identity of the associated protein. One possibility is that this protein may be the *pac*-cleaving protein, which is bound to one of the ends of the transducing DNA and injected into the recipient cell with it. Following transduction, the protein then associates with the other end to form a ring. The failure of association will result in digestion of transducing DNA by host nucleases or recombination into the chromosome (Yarmolinsky and Sternberg, review 1988). Another suggestion is that the *darA* operon plays a role in this process, because a deletion of that operon causes the Gta$^-$ (generalized transduction affected) phenotype – increasing the frequency of stable transduction and the Tsu$^-$ (transduction stimulated by ultraviolet irradiation) phenotype – insensitivity to stimulation by UV irradiation (Iida *et al.*, 1998). Other candidates are the products of *darA* and *darB* genes themselves, since Iida *et. al.* found that the products of *darA* and *darB*, which set up an antirestriction system, protect any DNA packaged into a phage head from restriction including transduced chromosomal markers. They do so by binding to the DNA and being injected into recipient cells along with it (Iida *et al.*, 1987). Since DarB is a rather big protein, up to 200kD, it would satisfy the observation by Ikeda and Tomisawa (1965b), that the total amount of protein bound to the transducing DNA is about 500kD. Studies on abortive transduction of Salmonella phage P22 showed that a mutation, called *tdx-1*, was responsible for reduced ability of P22 to form abortive transductants. The *tdx-1* mutation has been mapped to a region of the P22 genome that encodes several injected proteins and may involve more than one mutant locus (Benson, and Roth, 1997). Three products of genes 7, 16 and 20 are probably the candidates for this phenotype (Benson, and Roth, 1997). They are believed to be essential for the injection and/or protection of injected DNA and are probably coinjected with the DNA into the recipient host cell (Hoffman and Levine, 1975a, b; Israel, 1977). However, it seems unlikely for DarA and DarB to be proteins which are involved in abortively transduced DNA. Neither of these proteins is the product of the gene with the *sus*50 mutation in P1Cm*cts*-1*sus*50, since both proteins were found in purified heads isolated from a lysate of P1Cm*cts*-1*sus*50 (Iida *et al.*, 1987; Yamamoto, 1982). P1Cm*cts*-1*sus*50 showed the same two phenotypes, Gta$^-$ and Tsu$^-$, as the *darA* operon deletion mutant does and showed that the frequency of

stable transduction per infective phage appeared to be increased about 10-fold at the cost of a 10-fold decrease of that of abortive transduction (Yamamoto, 1982).

Table 2 Summary of the hypotheses about abortively transduced protein

Name	Evidence and hypotheses	Support	Against
Protein associated with transducing fragment Ref.: Ikeda and Tomizawa (1965a)	1. Proved transducing fragment is a DNA-protein complex by observing its buoyant density in CsCl and proteolytic enzymes. 2. M.W. of this protein is 500 KD. 3. The complex was stable to heating at 93°C for 10min and relative stable in alkaline buffer of pH 8.8 at 37°C for 60min, suggesting an ester linkage is involved in the DNA-protein complex.	**DNA binding protein**	
Sandri and Berger's AT Protein Ref.: Sandri and Berger (1980b)	1. Abortively transduced DNA appears to be held in a ring by a protein, for it has long-term stability. 2. In sucrose gradient and agarose gel, the mobility of abortively transduced DNA is similar to that of supercoiled and relaxed circular prophage DNA and 1.7-1.8 times faster than linear P1 phage DNA. 3. Abortively transduced DNA can be converted to a linear form by treating with sodium lauryl sulphate, heat(70°C) and Pronase, suggesting nonconvalent link.	**DNA binding protein found on AT DNA.**	**The protein is nonconvalently linked. This is inconsistent with Ikeda's finding.**
Yamamoto's *sus*50 mutant's product Ref.: Yamamoto (1982)	1. Produced by an amber mutantion named *sus*50, of phage P1. *sus*50 might be a single mutation. 2. In generalized transduction by *sus*50 mutant phage, the frequency of abortive transduction per infectious phage decreased to about 1/10 that of P1CM*cts*-1 *sus*$^+$ phage, while that of stable transduction appeared to be about 10 times higher than that of P1CM*cts*-1 *sus*$^+$ phage. 3. Stimulation of stable transduction by UV irradiation was not observed, suggesting the *sus50* protein is needed for abortive transduction	**The removal of this protein results in reduction of AT protein.**	

Name	Evidence and hypotheses	Support	Against
Pac-cleavage protein Ref.: Yarmolinsky and Sternberg (1988)-review	1. AT protein is the *pac*-cleavage protein, which is transferred from one "headful" to another as packaging proceeds along the P1 concatemer. 2. Speculated that processive packaging from a pre-existing end on the chromosome (as opposed to processive packaging from a cleaved *pac* site) does not involve a protein transfer step. Rather, the protein binds to the end and brings that DNA into an empty phage head after the "headful" cut occurs.	**Pacase is DNA binding protein and has affinity for DNA ends**	**Why should the protein be Pacase?**
Proteins from *darA* operon Ref.: Iida *et al.* (1998)	1. Deletion of *darA* operon showed Gta$^-$ and Tsu$^-$ phenotypes. 2. A complementary experiment showed that the genes affecting these phenotypes are likely to be different, because while Gta$^-$ can be partially complemented, the Tsu$^-$ phenotype cannot be complemented.	**Gta and Tsu phenotypes are thought to be related to abortive transduction.**	**No direct evidence**
DarA and DarB proteins Ref.: Iida *et al.* (1987)	1. They protect any DNA packaged into a phage head including transduced chromosomal markers from restriction by binding the DNA and are injected into recipient cells along with it. 2. Both of them are the so-called internal proteins. The protein attached to the end of a transducing fragment is supposed to come from one of such internal proteins. 3. Neither of these proteins, however, is the product of the gene with the *sus*50 mutation and neither DarA nor DarB seems the protein that Ikeda and Tomizawa found covalently attached to the end of transducing fragment, because both DarA and DarB proteins are found in infectious particles and because they seem to be easily removed from DNA by phenol treatment.	**1) binding to the DNA** **2) injected into the recipient along with the DNA** **3) DarB is about 200kD, close to the size estimated by Ikeda.**	**1) sus50 did not remove these proteins** **2) found in infectious particles** **3) inconsistent with Ikeda's finding, since they are not covalently attached to the DNA**

So far, the identity of the protein associated with transduced DNA remains obscure. Where does it come from? How does it interact with abortively transduced DNA and what is its function? The identity and origin of that protein still remains to be further studied. To identify abortively transduced protein was the aim of my initial project.

1.4. Transduction of plasmids by Coliphage P1

The analysis of generalized transduction has concentrated on the transduction of bacterial chromosomal DNA from donor to recipient cells. The DNA of extrachromosomal elements such as plasmids can also be transduced, but this process has been less well-studied. Generalized transducing phages, such as P22, T4, Mu, SPP1 and P1, package their DNA by the cutting of headful sized segments from a head-to-tail concatemeric precursor thought to be the product of rolling-circle replication. The chromosome, once packaging is initiated, can be packaged because it is longer than the phage genome. Large plasmids can be as long as phage genomes and thus transduced like chromosomes (Yarmolinsky and Sternberg, review pp406, 1988). However, shorter plasmids can also be transduced and the question is, how is this achieved?

Research on plasmid transduction by P22, the Salmonella phage, showed that cloning DNA homologous to the P22 genome into pBR322 could facilitate the transduction of the plasmid 100-1000 fold relative to that of pBR322 plasmid alone (Schmidt and Schmieger, 1984). Moreover, both the host recombination system, *recA*, and the phage recombination system, *erf*, influence the production of plasmid transducing particles. The transduction frequency of the plasmid from $recA^{+}/erf^{+}$ donors could vary from 100 to 1000 fold higher than that from $recA^{-}/erf^{-}$ strains. However, the *recA* system is apparently more efficient than the *erf* system. Surprisingly, it seems that the cloned P22 *pac* site appears to facilitate transduction of plasmid DNA even in the absence of homologous recombination systems, since only plasmids containing the P22 *pac* site can be transduced when the *rec* function of the donor cells and the phage *erf* system are simultaneously inactivated (Schmidt and Schmieger, 1984). Other work done on plasmid transduction by P22 showed that the transduction frequencies of plasmids are dependent on the size of the P22 DNA inserts in the plasmids and that transduction frequencies could vary from 10^{-4} to 10^{-2}. Also, *recA* plays an important part in plasmid transduction not only in donor cells, but also in recipient cells (Orbach and Jackson, 1982). To understand the mechanism of plasmid transduction by P22, Orbach and his collaborator used restriction endonucleases to analyze the transducing lysate DNA and the electron microscope to observe heteroduplexes between transducing particle DNA and P22 DNA. They demonstrated that the

transduction of the chimeric plasmids involves two homologous recombination events. In the donor cell, a single recombination between phage genome and the insert in the plasmid allows the plasmid to be integrated into the phage genome and packaged by headfuls into the P22 capsid. Once the plasmid transducing DNA gets into the recipient cell, a second recombination between the duplicated regions flanking the plasmid regenerates the plasmid. However, work in Schmidt's lab also demonstrated that in addition to the above cointegrate mechanism, transduction of pBR322 by P22, which requires concatemer formation of the plasmid DNA, can also occur (Orbach and Jackson, 1982; Schmidt and Schmieger, 1984).

Another well-studied phage which is able to transduce is coliphage T4. Mattson and his collaborators found that the replication of plasmid pBR322 containing T4 DNA could be resumed after bacteriophage T4 infection, while the plasmid pBR322 replication would be inhibited after T4 infection (Mattson *et al.* 1983a). This result suggested that the T4 homology-dependent synthesis of plasmid DNA is due to recombination between plasmids and phage genomes. Further work (Mattson *et al.* 1983b) detected labelled plasmid DNA in T4 progeny particles. Use of Southern blotting to analyze the restriction endonuclease-digested phage DNA isolated from mature phage particles, supported the idea that recombination between cloned T4 DNA sequences in the pBR322 plasmids and homologous sequences in the T4 genomes leads to integration of the entire plasmid into the phage genomes. However, two Japanese biologists, Takahashi and Saito, demonstrated that a T4 mutant called T4dC, which, instead of glucosylated hydroxymethylcytosine (glu-HMC), incorporated cytosine into its DNA, could transduce plasmid pBR322 without any cloned T4 DNA fragments (Takahashi and Saito, 1982). To analyse the DNA in the transducing particles, they further demonstrated that the form of pBR322 in the phage head after packaging is actually head-to-tail multimers, although the plasmids could return to CCC monomers in the recipient cells, in which case the *recA* function plays an essential role in this interconversion.

Some work on transduction of plasmids by phage lambda, Mu and SPP1 has also been reported. Phage Mu can transduce plasmid pBR322 as a head-to-tail oligomer which will be circularized in the recipient cell (Teifel-Greding, 1984). The *rec* system is essential for the completion of the Mu-mediated transduction process in the recipient cell, since no transductants appeared when a *rec*$^-$ strain was used as the recipient. However, the *rec* system in the donor cell does not seem essential, although lysates made from a *rec*$^-$ donor strain resulted in a 10-fold decrease in the transduction frequency of

the plasmid markers, although transduction of chromosomal markers is not influenced by the *rec* system of the donor strain. It is likely that rolling circle replication plays a role in the formation of pBR322 multimers in Mu-infected cells as it does in phage lambda-infected cells (Umene, *et al.*, 1978), while the *rec* system of the donor cell could possibly enhance the transduction frequency by conducting homologous interplasmid recombination. SPP1, a *Bacillus subtillis* phage, is another well-studied phage which can transduce plasmid DNA in a head-to-tail form. When cloned in plasmid pBR322 the phage DNA could enhance the transduction frequency of the plasmid by 100- to 1000-fold, however, in contrast to P22 and other coliphages, this increase is independent of the size of the cloned phage fragment and also independent of the host *rec* system both in the donor and in the recipient cell (Deichelbohrer, *et al.*, 1985). To explain what is happening between phage SPP1 and the homologue in the plasmid, Alonso and his collaborators (Alonso, *et al.* 1986) put forth a model (Fig. 10), in which a synaptic structure is formed between the homologous sequences in the plasmid and phage. This structure allows the phage rolling circle replication machinery to be shared by both phage and plasmid DNA to generate long DNA concatemers.

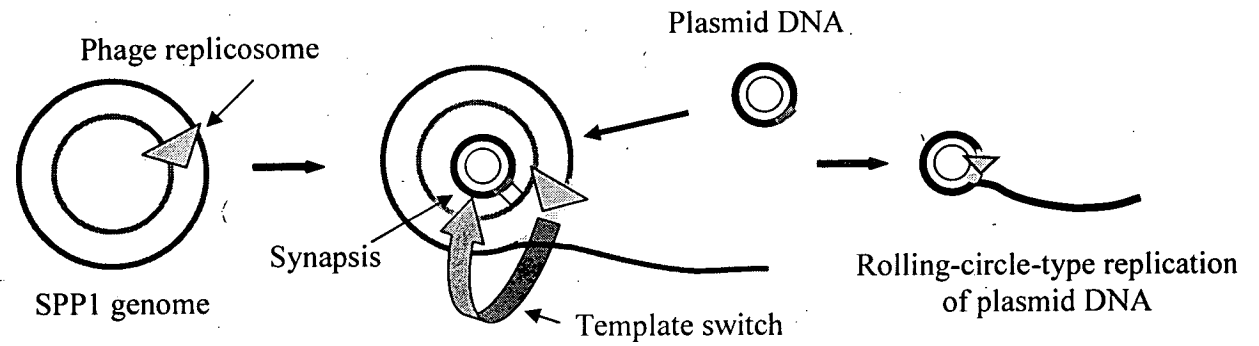


Fig 10 A proposed model for the generation of concatemeric plasmid DNA. A synapsis between phage and plasmid is formed at the homologous segments, which allows a switch of the template from phage genome to plasmid and therefore leads the plasmid to undergo rolling-circle replication using phage-specific DNA replication machinery. (Alonso, *et al.* 1986)

Table 3 Summary on plasmid transduction by different phages.

es	Plasmid (pBR322 based) transduction						
	Homology to phage DNA (***Hph***)	*pac* site affect	Homology to chromo. (***Hch***)	Donor *RecA*	Recipient *RecA*	Recomb. into either kind of chromo.	Plasmid DNA concater.
reported	**Required** (if no ***Hch***) frag. size-independent ?	**No**	**Required** (if no ***Hph***) fragment - independent	?	**Needed**	**Yes** could *(Iida et al., 1981)*	**Yes** Probably *(O'Conner, 1983)*
	Required to facilitate; Frag. size - dependent	**Yes** *(Schmidt et al., 1984)*	?	**Needed to promote**	**Needed to promote**	**Yes** *(Schmidt et al., 1984)*	**Yes** *(Orbach et al., 1982)*
WT	**Required** fragment - independent	?	?	?	?	**Yes** *(Mattson et al., 1983a, b)*	? probably not
T4dC	**Not required**	?	**Not required**	? intramolecular recombine.	**Needed**	**No** *(Takahashi et al., 1982)*	**Yes** *(Takahashi et al., 1982)*
reding et 984)	**Not required**	?	**Not required**	**Needed to promote** other pathways?	**Needed**	**No** *(T.-Greding et al., 1984)*	**Yes** *(T.-Greding et al., 1984)*
bda *ne K et 978)*	**Required**	**Cosλ required**	**Not required**	**No need**	**No need**	?	**Yes**
helbohrer 1985) so et al.,	**Required** to facilitate; Frag. size - independent	?	?	**No need**	**No need**	**No**	**Yes**

Note: "?" means "not tested" or "not clear".

Table 5.1 is a short summary on plasmid transduction by different phages. So far, regarding plasmid transduction, little work has been done on phage P1. Therefore, whether P1 can transduce plasmid as other phages do and how it works still remains unclear. In chapter V, we have taken a

glance at the nature of plasmid transduction by P1, though there are still many questions left to be answered and further work is needed.

Although few studies have been done on P1 plasmid transduction, some hypotheses have been proposed in previous work. In 1981, Iida and his colleagues demonstrated that small plasmids, such as pBR322 derivatives could be transduced by P1 via co-integration with P1 DNA. An IS*1* element is required for the P1 cointegrate formation and the transductants appeared to be P1 immune (Iida *et al.*, 1981). O'Connor and Zusman (1983) worked on transduction of relatively large plasmids from *E. coli* to *Myxococcus xanthus* using P1. The plasmids contain a sequence from *M. xanthus* and a Tn5 (kan^r) insert, but no sequence from *E. coli*. They found the *E. coli* plasmid-transductants to be indistinguishable from transformed plasmid-bearing *E. coli* since the transductants do not become P1 immune and are not inducible for P1. The restriction digest patterns of plasmids isolated from the transductants are also identical to those from transformants. These observations imply that the plasmids are not transduced as stable cointegrates in the P1 genome. In addition, they also found that *recA* function is necessary for establishment of the plasmid in the recipient cells and that IS*1* element does not play a role in the packaging of these plasmids (O'Connor and Zusman, 1983). From the above observations, they proposed two mechanisms involved in the packaging of the plasmid by P1. One possibility is that cointegration of plasmid multimers and P1 DNA occurs to form a DNA substrate for packaging longer than a "headful" and allows the packaging to begin at the normal P1 *pac* site. The packaged DNA lacks terminal redundancy and cannot cyclize when injected into the recipient cell but it can generate circular plasmid DNA by excision of a plasmid monomer from the injected linear DNA using the recipient homologous recombination system, such as the *rec* system. The other possibility is that plasmid multimers without P1 DNA involved are formed and directly packaged into the P1 heads at random as is chromosomal DNA. The latter hypothesis is supported by another finding that the efficiency of transduction is proportional to the size of the plasmids. To account for this, O'Connor and Zusman (1983) proposed that dimers or multimers have to be made for the plasmids whose size is smaller than 100kb in order to form a substrate long enough for headful packaging. Therefore, the smaller the plasmid is, the more difficult to form the substrate since formation of dimers occurs in higher frequency than other higher-order multimers.

Previous work in our lab shows (Sean McAteer, personal communication) that cloned chromosomal DNA fragments in the plasmid pUC18 could increase the P1 transduction frequency of the plasmid markers by 100-fold. The transduction is also *rec*-dependent in the recipient cell. For plasmids without homology to the chromosome, no transductants could be observed in *recA*$^-$ recipients, while there are always about 20 transductants that appear in *recA*$^-$ recipients for homology-containing plasmids, 40-fold less than in *recA*$^+$ recipient cells. Since this observation is reproducible, it possibly indicates that the host *rec* system in the recipient cell is the main recombination system for the re-formation of the transduced plasmids, but not the only one. Furthermore, Southern blots, using P1 DNA purified from lysates made from plasmid-bearing strains, demonstrated that when the plasmid was digested by bglII, which does not cut the plasmid, a huge band equal to dozen of single plasmids appeared, while only one single plasmid sized band appeared when HindIII, which cuts the plasmid once, was used to perform the digestion. This result showed that the P1 transducing particles appear to contain exclusively plasmid oligomers, suggesting that instead of having been integrated into the chromosome, plasmids form an exclusively plasmid multimer before being packaged into the P1 capsid. To further understand how plasmids are transduced by P1, more work is needed.

1.5 Aims of this thesis

The experiments presented in Chapters 3-5 of this thesis have been involved in two main separated projects. In the first project, experiments were carried out to investigate the nature of abortively transduced DNA and protein. A wide approach in order to purify abortively transduced DNA and protein was utilized, meanwhile, *darA* operon, which has been reported to be involved in abortive transduction, was also studied. In the second project, the mechanism of packaging chromosomal DNA at the *pac* sites was studied by adding up to 5 *pac* sites into the chromosome. Massive molecular biological approaches as well as physiological and genetic experiments have been done for this purpose. In addition, transduction of plasmid by P1 has also been studied.

Chapter II

Materials and Methods

2.1. Bacterial, Phage P1 strains and plasmids

Bacterial strains are listed in Table 2.1. Bacteria were maintained either in Bijous stored at room temperate or on nutrient agar plates stored at 4 °C for regular use. Stocks were made by mixing frozen buffer, consisting of 50% bacterial buffer and 50% glycerol, with an equal volume of overnight culture and then stored at -70 °C.

Bacteriophage P1 that I used in this work is P1kc from our laboratory stock, which is supposed to produce more transducing particles, up to 2% of the total lysates, during every round of infection. The phage P1 had been purified from a single plaque before use for titration and transduction and gave a titre of 1 x 10^9 pfu/ml. Normally, P1 was stored in either L broth or phage buffer with supply of 2 x 10^{-3} M Ca^{2+}.

Table 2.1. Bacterial strains

Strain No.	Strain Name	Genotype	Source /Reference
1	AB352(purF)	thr-1 leuB6 lacZ4 supE44 purF-1 rpsL8 thi-1 λ^s mal^+	Lab stock
2	DH5α	supE44 ΔlacU169(ϕ80 lacZΔm15) hsdR17 endA1 gyrA96 Thi-1 relA1	Lab stock
3	GIA39	thr-1 dadB3 leuB6(Am) flmA21 codA1 LacY1 tsx-95 glnV44(AS) λ^- pyrF101 His-108 argG6 *ilv*A634 thi-1 deoC1 glt-1S	Lab stock
4	MG1655	wild type,	F. Blattner
5	**MG1655(1-arg)**	(argB<>*pac*) – 90min	This work

6	**MG1655(1-pb)**	(*pac* ig, phoR-brnQ) – 9min Km^R(FRT-Kan cassette)	This work
7	**MG1655(1-de)**	(*pac* ig, dsdA-emrY) – 54min Km^R(FRT-Kan cassette)	This work
8	**MG1655(1-dy)**	(*pac* ig, dacB-yhbZ) – 75min Km^R(FRT-Kan cassette)	This work
9	**MG1655(1-tt)**	(*pac* ig, tyrR-tpX) – 29min Km^R(FRT-Kan cassette)	This work
10	**MG1655(2pac)**	*pac* positions referred to 5 and 6 – 90min, 9min Km^R(FRT-Kan cassette)	This work
11	**MG1655(3-de)**	*pac* positions referred to 5, 6 and 7 – 90min, 9min, 54min Km^R(FRT-Kan cassette)	This work
12	**MG1655(3-dy)**	*pac* positions referred to 5, 6 and 8 – 90min, 9min, 75min Km^R(FRT-Kan cassette)	This work
13	**MG1655(3-tt)**	*pac* positions referred to 5, 6, and 9 – 90min, 9min, 29min Km^R(FRT-Kan cassette)	This work
14	**MG1655(4pac)**	*pac* positions referred to 5, 6, 8 and 9 – 90min, 9min, 75min, 29min Km^R(FRT-Kan cassette)	This work
15	**MG1655(5pac)**	all *pac* positions as described above Km^R(FRT-Kan cassette)	This work
16	**MG1655(5pac, kan$^-$)**	all *pac* positions as described above Km^s	This work

17	MM18	F^- argG6 asnA31 asnB32 hisG1 leuB6 *metB*1 pyrE gal-6 lacY xyl-7 supE44 bgl^+ fhuA2 T1R ph80R gyrA NalR rpsL104 strR srl^- T6R uhp^- lmb^-	Lab stock
18	MM303	F^- DEL *trp*-tonB argH1 hisG1 *ilv*-192 *metB*1 pyrE malA1(λ^R) lac y1/Z4 xyl-7 thi-1 supE44 rpsL8/9/17(strR) tnaA1 tsx-7($T6^R$) ahp^- (P_1)	Lab stock
19	**W3110(thy^-)**	Thy^- isolated by TmpR selection	Lab stock

Note: 1, the orientation of the pac sites on the pac-containing strains are showed on the map (Fig 4.8). 2, lysates made from pac-containing strains are referred to the* strains' *name. 3, FRT-Kan cassette present in the pac-containing strains is referred to plasmid pTOF3.

Table 2.2. Plasmids

plasmid	Description	Source/Reference
pBR322	Amp^R; Tet^R; pMB1 replicon	Bolivar 1978
pGEM-T	from pGEM-5Zf(+) by cutting with EcoR V and adding a 3'-terminal thymidine (T) to both ends.	Promega
pGB2	pGEM-T derivative containing argCH gene crossover PCR fragment	This work
pHM	pTOF24 Δ[PstI-SalI: 1293bp], Ω[PstI-SalI: polylinker, PstI-BamHI-PacI -SacI-SmaI-SwaI-NheI -NotI-AscI-SalI] Cm^R Ts Suc^S	This work
pHP19	pHM Ω[EcoRI-EcoRI: 695bp, P1 EcoRI 20 fragment, containing the *pac* site, from pPAC] Cm^R Ts Suc^S	This work

pHPKAN	pHP19 Ω[NotI-NotI: 1414bp, NotI-FRT-KAN -FRT-NotI] Cm^R Km^R Ts Suc^S	This work
pHPK-brn	pHPKAN Ω[NotI-SalI: 609bp, brnQ gene PCR fragment] Cm^R Km^R Ts Suc^S	This work
pHPK-pb	pHPK-brn Ω[BamHI-SacI: 572bp, phoR gene PCR fragment] Cm^R Km^R Ts Suc^S	This work
pHPK-dsd	pHPKAN Ω[BamHI-SacI: 588bp, dsdA gene PCR fragment] Cm^R Km^R Ts Suc^S	This work
pHPK-de	pHPK-dsd Ω[NheI-AscI: 597bp, emrY gene PCR fragment] Cm^R Km^R Ts Suc^S	This work
pHPK-tyr	pHPKAN Ω[BamHI-SacI: 552bp, tyrR gene PCR fragment] Cm^R Km^R Ts Suc^S	This work
pHPK-tt	pHPK-tyr Ω[NotI-SalI: 530bp, tpX gene PCR fragment] Cm^R Km^R Ts Suc^S	This work
pHPK-yhb	pHPKAN Ω[NotI-SalI: 597bp, yhbZ gene PCR fragment] Cm^R Km^R Ts Suc^S	This work
pHPK-dy	pHPK-yhb Ω[BamHI-SacI: 596bp, dacB gene PCR fragment] Cm^R Km^R Ts Suc^S	This work
pJF118EH	pKK223-3 Δ[XmaIII-BamHI: 0.5kb], Ω[bla, lacIQ, tacP, PstI-EcoRI, 4.8kb], Ap^R, Sm^R	(Gene, 48: 119-131)
pJF118-his	based on pJF118EH, but replaced multiple cloning sites with a new linker : EcoRI-SalI-BamHI-PstI-His taq-HindIII	This work
pKO3	Cm^R; pSC101-based vecor; repA(Ts) with a sacB conferring sucrose sensitivity	Church lab (JB.179)

pHM2	pKO3 derivative containing argCH gene crossover PCR fragment	This work
pHM3	pKO3 derivative containing argCH gene crossover PCR fragment and the *pac* site fragment cut from pPAC	This work
pPAC	pBR325 derivative, containing P1 genome EcoRI 20 fragment which contains the *pac* site	Mark C. Hanks
pTOF1	pUC18 Ω [EcoRI-HindIII: 162bp, NotI-FRT-SmaI-FRT-NotI]; Ap^R	Dr. Merlin (JB.184.16)
pTOF3	pTOF1 Ω [HincII-HincII: 1252 bp, aph gene from pUC4K]; Ap^R Km^R	Dr. Merlin (JB.184.16)
pTOF24	pKO3 Ω [HincII-HincII: 1252 bp, aph gene from pUC4K]; Cm^R Km^R Ts Suc^S	Dr. Merlin (JB.184.16)
pSHI93-Δ4	*darA*+; lyd+; cin+; constracted by a partial digestion of pSHI93 with EcoRI followed by religation, containing a big fragment of P1 *darA* operon	(Virology 251 :49-58)

2.2. Growth media and buffers

L broth and agar were usually used for bacterial growth and making P1 lysates. BBL bottom and top were used for phage titration and VB minimal agar used routinely for testing P1 transduction. For all phage P1 manipulations, 2.5mM CaCl was added to the medium, either to allow cells to be able to absorb phage P1 or to stablize phage P1 in the presence of $CHCl_3$.

2.2.1. Growth Media

L-broth

Difco bacto tryptone	10 g
Difco bacto yeast extract	5 g
Nacl	10 g
pH to 7.2 with NaOH	
Distilled water to 1 litre	

L-agar

L-broth + 15g Difco agar per litre

LC top agar

Difco bacto tryptone	10 g
Difco bacto yeast extract	5 g
Nacl	5 g
Difco Agar	7 g
Dist. Water	to 1 lit.

BBL bottom agar

BBL trypticase	10g
NaCl	5g
DIFCO AGAR	10g

Dispense in 500ml & 250 ml amounts.
Autoclave 121°C for 15 minutes.

BBL top agar

BBL trypticase	10g
NaCl	5g
DIFCO AGAR	6.5g

Dispence in 100ml.
Autoclave 121°C for 15 minutes.

VB minimal media

20x VB salts	25 ml
20% carbon source	5 ml
Supplements as required	
Distilled water to 1 litre	

VB minimal agar

VB minimal media + 15g Difco agar per litre

20x VB salts	$MgSO_4.7H_2O$	4 g
	Citric acid	40 g
	KH_2PO_4	400g
	$NaNH_4HPO_4.4H_2O$	70 g
	Distilled water to 1 litre	

TCG minimal medium	Tris-HCl pH 7.4	0.1M
	Na_2SO_4	0.16mM
	$FeSO_4$	2×10^{-3}mM
	$MgSO_4$	1.0mM
	NaCl	8.6mM
	$CaCl_2$	0.1mM
	Thiamine	2 μg/ml
	Glucose	0.25%
	Casamino acids	0.5%

(add KH2PO4 to a final concentration of 20μg/ml and 1μg/ml thymine, 5μg/ml BU added)

2.2.2. Commonly used buffers

TAE buffer

Working solution:

40 mM Tris-acetate
2 mM EDTA

50x Conc. Stock solution:

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Distilled water	to 1 litre

TBE buffer

Working solution:

89 mM Tris-borate
89 mM boric acid

50x Conc. Stock solution:

Tris base	54 g
Broc acid	27.5 g
0.05 M EDTA (pH 8.0)	20 ml
Distilled water	to 1 litre

TE buffer

10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

TELS buffer

0.05 M Tris-HCl (pH 8.0)
0.005 M EDTA
0.05 M NaCl

Phage buffer

Na_2HPO_4	7 g
KH_2PO_4	3 g
NaCl	5 g
$MgSO_4$ (0.1M)	10 ml
$CaCl_2$ (0.1 M)	10 ml
1% gelatine solution	1 ml
Distilled water to 1 litre	

Bacterial buffer

KH_2PO_4	3 g
Na_2HPO_4	7 g
NaCl	4 g
$MgSO_4.7H2O$	2 g
Distilled water	to 1 litre

Hybridization solution

1 M NaPO4 buffer	10 ml
20% SDS	7 ml
H_2O(TE)	3 ml
	Total: 20 ml

10x ExoV reaction buffer (*J. Biol. Chem.* 272(15) P10072-)	imidazole	430mM
	Hepes-NaOH (pH=7.5)	350mM
	MgCl2	10mM
	DTT	6.7mM

2.2.3. Selection of Antibiotic Resistance

Information about the antibiotics used in this work has been listed below:

Table 2.3. Information on antibiotics used in this work

Antibiotic	Abbreviation	Solvent	Method of sterilization	Concentration	
				Stock (mg ml^{-1})	Final (μg ml^{-1})
Ampicillin	Amp	H_2O	Filtration	100	50-100
Chloramphenicol	Chl or Cmp	ethanol	_____	20	20
Kanamycin sulphate	Kan	H_2O	Filtration	25	20-50
Spectinomycin dihydrochloride	Spc	H_2O	Filtration	50	0.5
Tetracycline hydrochloride	Tet	50% ethanol	_____	10	10
Trimethoprim	Tmp	methanol	_____	5	20-50

2.2.4. Expression supplements

X-gal and IPTG were used to distinguish between lac^+ and lac^- by the colour blue-white test, while IPTG was used as an inducer of expression in the pJF118-his plasmid.

Table 2.4. Information of other selection agents in this work

Selection Agent	Abbreviation	Solvent	Method of sterilization	Concentration	
				Stock (mg ml^{-1})	Final (μg ml^{-1})
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside	X-gal	Dimethyl-formamide	____	20	20-40; 84mM - plasmid induction
Isopropyl-β-D-thiogalactopyranoside	IPTG	H_2O	____	20	20

2.3. Bacterial and phage techniques

Bacteria were routinely grown either as liquid cultures at 37°C, or on agar-plates at 37°C (30°C for temperature-sensitive strains). Usually fresh overnight cultures were inoculated for making liquid cultures the next day. For selection of strains having pKO3-based plasmids integrated into chromosome, cells would be grown on agar plates at 42°C.

2.3.1 Bacterial techniques

2.3.1.1. Growing cells for growth curves

Overnight cultures were diluted 50-fold into 25ml of LB in 250ml flasks and shaken at 37°C in a water bath. 500µl aliquots were taken every 20 minutes and the OD_{600} was measured using a UV spectrophotometer (HITACHI U-2000). Once the OD_{600} value reached 0.3-0.4, the cultures were then 5-fold re-diluted into 20ml of fresh LB. The procedure was repeated at least twice.

2.3.1.2. Preparation of competent cells using chemicals and transformation with plasmid DNA

A fresh overnight culture of the strain was used to inoculate LB by diluting 1:50 (v/v), and incubated at 37°C (or 30°C for temperature sensitive strains) with vigorous shaking until the OD_{540} was between 0.5 – 0.6. The culture was chilled on ice for 10 min, transferred to sterile centrifuge bottles and pelleted at 5000rpm in a Sorvall GSA or G3 rotor for 10 min at 4°C or at a maximum speed of a bench centrifuge in BIJOU/universal for 5 min at room temperature. The supernatant was discarded and cells were resuspended in 1/2 the original culture volume of ice-cold 0.1M $MgCl_2$. The bacterial pellet can be efficiently resuspended by sucking in and out of a pipette several times. Then the cells were pelleted again. The supernatant was poured off and cells were resuspended in 1/20 the

original volume of ice-cold 0.1M $CaCl_2$. The cell suspension was transferred to a sterile universal bottle and kept on ice for more than 2 hours. 0.2ml and 0.1ml samples were taken into labelled, sterile 1.5ml Eppendorf tubes and the tubes were placed on ice ready for transformation.

To carry out transformation, plasmid DNA was added to each tube above. The contents of the tubes were mixed by swirling gently and the tubes sat on ice for 30 min. Then the tubes were placed at 42°C for 90sec without shaking and rapidly transferred to an ice bath and chilled for 1-2min. 800µl of LB was added to each tube and the tubes were gently agitated at 37°C for 45min. 0.1ml transformed competent cells were transferred to the plates and spread. Plates are then incubated at 37°C (or 30°C for temperature sensitive strains) overnight.

2.3.1.3. Preparation of competent cells for electro-transformation and electro-transformation with DNA

25ml of LB was inoculated with 1/100 volume of fresh overnight culture. Cells were grown at 37°C with shaking to an OD_{600} of 0.5. The flask was chilled on ice for 15-30min and then centrifuged with a bench centrifuge for 5min. The pellet was resuspended in 25ml of cold water and centrifuged as above. Then the pellet was resuspended in 15ml of cold water and centrifuged again. The pellet was resuspended in 1ml of cold water and centrifuged again as described above. Finally cells were resuspended to a final volume of 50µl in cold water. The cell concentration should be about 3×10^{10} cells/ml.

To transform DNA into the cells, 50µl of cells were mixed with 0.5µl (not more) of uncleaned ligation mixture or plasmid DNA. Ideally, DNA should be in low ionic strength buffer such as TE or water. 40µl of the mixture was transferred into a cold 0.1cm electroporation cuvette and shaken to the bottom. There should be no air bubbles. Let it sit on ice for about 1min. The gene pulser apparatus was set at 25µF and 1.8KV for 0.1cm cuvettes, and the pulse controller was set to 200Ω. The cuvette was placed in a safety chamber slide, and the slide was pushed into the chamber until the cuvette was seated between the contacts in the base of the chamber. Then both buttons were pressed till a beeping noise could be heard. This should produce a pulse with a time constant of 4.5 to 5ms. The cuvette was

removed from the chamber and 1ml of SOC medium or LB + 0.4% glucose was immediately added to resuspend cells. The cell suspension was then transferred into an eppendorf tube, and incubated at 37°C for 1 hour with shaking. Finally, cells were spread on selective plates and incubated at 37°C (or 30°C for temperature sensitive strains) overnight.

2.3.2. Phage P1 techniques

2.3.2.1. Preparation of phage P1 liquid lysates (1) (without wash and resuspension step – infection not synchronous)

An overnight culture of a P1 sensitive strain, such as Mg1655 or W3110, was diluted 50-fold into L Broth with 2.5 x 10^{-3} M Ca^{2+} in a flask of a capacity at least 5-fold greater than the volume of the medium and grown at 37°C with vigorously shaking. Once the culture was grown to an OD_{600} of 0.4-0.5, the culture was then infected with P1 at a m.o.i. of about 2. Incubation was continued until lysis. Then the lysis was completed by adding chloroform into the culture and incubation was continued for a further 15min. Lysates were then centrifuged at 10,000 rpm in a GSA rotor of a Sorvall RC-5B centrifuge to remove cell debris. The supernatant was kept as P1 stock at 4°C in presence of chloroform.

2.3.2.2. Preparation of phage P1 plate lysates (2)

An overnight culture of a P1 sensitive strain, such as Mg1655 or W3110, was diluted 50-fold into 5 ml of L Broth in the presence of 2.5 x 10^{-3} M Ca^{2+} in a flask of 50 ml and grown at 37°C with vigorously shaking, to an OD_{600} of 1-1.5. Then 1 ml of the above culture was infected with 10^6 of phage P1 and incubated at 37°C for 15min without shaking. After incubation, 3 ml of LB followed by 3 ml of LC top agar both in the presence of 2.5 x 10^{-3} M Ca^{2+} was added into the culture and the

mixture was then poured onto the LB plates, and incubated at 37°C overnight. Top agar was transferred into a sterile 125 ml beaker using a spreader the next day and plates were washed with 5ml of LB in the presence of 2.5 x 10^{-3} M Ca^{2+}, and then the wash was transferred into the same beaker. 0.5 ml of $CHCl_3$ was added into the beaker, which was then shaken at 30°C for 20min. The content of the beaker was transferred into a sterile universal bottle and then spun down at 4000 rpm for 15min. The supernatant was then kept in the presence of 2.5 x 10^{-3} M Ca^{2+} and chloroform as P1 stock and stored at 4°C.

2.3.2.3. Measurement of P1 single cycle lysis curve –single cycle lysate (3) (wash and resuspension step added to ensure synchronous infection)

As described above in 2.3.1.1, overnight cultures were diluted 50-fold into 25 ml of LB containing 2.5 x 10^{-3} M Ca^{2+} in 250 ml flasks and shaken at 37°C in a water bath, to an OD_{600} of 0.3-0.4, then 2 x 10^{-3} M KCN was added and followed by infection with phage P1 at a m.o.i. of about 4. Incubation was continued for 10min, by which time, 5 ml of cultures were taken and washed once with fresh LB to remove KCN and Ca^{2+}, then diluted 10-fold into 25 ml of fresh pre-warmed LB without Ca^{2+} in 250 ml flasks and shaken at 37°C in the water bath until lysis. After wash and resuspension, OD was measured every 10min and followed by sampling if subsequent experiments, such as titration and transduction, were required, otherwise measured every 20min after the wash. If separation of pellet and supernatant of the cultures was required, the aliquots were put in ice immediately after taking from the culture, then spun at 16,000g in a micro centrifuge. Supernatants were then chloroformed immediately, whereas pellets were washed with wash buffer (4g/100ml NaCl, 0.1%Tween and 20ng/ml Cmp), resuspended in phage buffer and then chloroformed.

2.3.2.4. Spot titration of phage P1 lysates

A strain, which is sensitive to P1, was used to make a cell lawn on a BBL plate by mixing 0.1 ml of cells with 2.5-3 ml of top BBL agar containing 2.5 x 10^{-3} M Ca^{2+} and pouring the mixture onto the plate. Serially, Phage P1 lysates were diluted 10^1, 10^2, 10^3-fold, *etc.* in small tubes and 10 µl of the serial dilutions were spotted on the cell lawn on corresponding numbers which had been marked on the plate beforehand. The plate was then incubated at 37°C overnight.

2.3.2.5. Plate titration of phage P1 lysates

P1 lysates were serially diluted 10^1, 10^2, 10^3-fold, *etc.* in small tubes and 10 µl of the dilutions were taken to mix with 0.1 ml of cells, which were sensitive to P1 and had been grown at 37°C to an OD_{600} of 1-1.5. The mixture was incubated at 37°C for 15min, then mixed with 2.5 ml of top BBL agar containing 2.5 x 10^{-3} M Ca^{2+} and poured onto BBL plates. Plates were incubated at 37°C overnight.

2.3.2.6. Transduction of phage P1 lysates

Recipient cells were grown in LB in the presence of 2.5 x 10^{-3} M Ca^{2+} at 37°C in a water bath with vigorously shaking to an OD_{600} of 1-1.5. To 100 µl of cells which are P1 lysogen, 10 µl to 100 µl of phage P1 was added, depending on the titer of the lysate (if the recipient is not P1 lysogen, cells have to be 10-fold concentrated before infection with P1), then the mixture was incubated at 37°C for 15min. Afterwards, if the selection marker is auxotrophic, 1 ml of phage buffer was then added into the tubes and 100 µl of aliquots were plated out on suitable plates. However, if the selection agent was an antibiotic, 1 ml of phage buffer was added into the tubes, and cells were spun down, resuspended in 1 ml of LB + glucose (10 ml LBG = 9.82 ml of LB + 0.18 ml of 20% glucose), and incubated at 37°C for 1 hour and 100 µl of aliquots were then plated out and incubated at 37°C overnight.

2.3.2.7. Preparation of heavy-light P1 lysates by labelling chromosomal DNA with Bromouracil (BU)

The procedure is modified from Sandri and Berger's work (1980). *E. coli* strain W3110, thy^- was grown overnight at 37°C in TCG medium with 20 µg/ml P (as KH_2PO_4) provided to which 5 µg/ml thymine had been added. The culture was diluted 50-fold into 300 ml TCG medium containing 5 µg/ml BU and 1 µg/ml thymine. The bacteria were incubated with vigorous shaking at 37°C to an OD_{600} of about 0.5. At this time, thymine was added to a final concentration of 50 µg/ml. The bacteria were incubated for an additional 10min, and then 2 x 10^{-3} M KCN and 5 x 10^{-3} M $CaCl_2$ were added. After 3min, P1 was added to a multiplicity of infection of 5 and allowed to absorb for 20min. The infected cells were centrifuged, resuspended in 500 ml of pre-warmed LB, and vigorously shaken at 37°C for about 90min by which time the culture had cleared. Chloroform was added, and the bacterial

debris was removed by centrifugation at 10,000 g for 10min. Phage were collected either by centrifugation at 13,000 g in a GSA rotor for 3hr, followed by re-suspension in phage buffer at 4°C overnight or by storing at 4 °C overnight in the presence of 10% PEG 8000, 40 g/L NaCl , centrifugation at 13,000 rpm in a GSA rotor for 30min and re-suspension in phage buffer at 4°C ove ght with a rotary shaker.

2.3.2.8. Transduction of CsCl purified BU labelled transducing particles to recipient cells

The procedure was a modification of the method described by Sandri and Berger (1980). Recipient bacteria were grown in LB containing 5 x 10^{-3} M Ca^{2+} at 37°C with vigorously shaking to an OD_{600} of 1-1.5. The bacteria were incubated at 4°C for 4 hours and then 4-fold concentrated prior to use. An appropriate dilution of CsCl gradient fractions containing transducing particles was added to recipient bacteria at a m.o.i. of about 0.5 and allowed to adsorb for 20min at 37°C. $CaCl_2$ was supplemented to a final concentration of 5 x 10^{-3} M.

2.3.2.9. Lysis of infected bacteria by Triton X-100

As described by Sandri and Berger (1980), recipient bacteria were infected with BU labelled transducing particles as described above. Infected bacteria were diluted into an equal volume of TELS buffer and centrifuged at 10,000 g for 10min at 4°C, then resuspended and re-centrifuged twice in 10 ml of TELS buffer. The bacterial pellet was resuspended in 1 ml of 25% sucrose in 0.05 M Tris-HCl (pH 8.0). The suspension was placed in an ice bath and maintained on ice throughout the following operations. After 5min, 0.2 ml of a freshly prepared lysozyme solution (10 mg/ml in 0.25 M Tris-HCl, pH 8.0) was added. After an additional 5min, RNase was added to a final concentration of 100 µg/ml. Again after 5min, 0.4 ml of 0.25 M EDTA (pH 8.0) was added, and the incubation was continued for 5min. The bacteria were lysed by the addition of 1.6 ml of a solution containing 0.2% Triton X-100, 0.0625 M EDTA, and 0.05 M Tris-HCl (pH 8.0). The suspension cleared in 20-30min. The lysates were centrifuged at 30, 000 g in an SS-34 rotor for 15min at 4°C to sediment the chromosomal DNA.

2.3.3. UV irradiation

The procedure was described by Barbara Newman in her thesis (1982). Phage suspensions were diluted 10-fold (about 10^9pfu/ml in this work) in phage buffer and placed in a 10 cm diameter glass Petri dish. The open dish was exposed to UV at a dose rate of 10 ergs/mm^2/second with agitation. Irradiated phage were shielded from visible light and used as soon as possible after irradiation. Transductions and titrations using irradiated phages were accomplished with the minimum exposure to light, and incubated in the dark to prevent photo reactivation.

2.3.4. Separation of BU-labelled transducing particles from infectious particles with CsCl equilibrium gradients

The procedure was modified from Sandri and Berger's (1980) and Ikeda and Tomizawa's (1965a). P1 transducing DNA has been labelled by BU as described above. 2 ml of P1 lysate in phage buffer was gently mixed with 1 ml of 1.9 g/cm^3 CsCl dissolved in phage buffer, and then the refractive index adjusted to 1.3790 by adding about 1 g CsCl. The phage was then centrifuged in an SW55 Ti rotor at 21.6K rpm for 20hr. at 20°C. Fractions were taken by puncturing a hole at the bottom of the tube and collecting the drops. Each fraction contained two drops, and then was diluted to a final volume of 300 µl with phage buffer.

2.3.5. Revised protocol of extraction of Abortively Transduced DNA

P1 heavy-light lysates (in which transducing DNA has been labelled by BU) were made as described above. 5 ml of MG1655 culture was grown at 37°C overnight, then diluted 50-fold into 200ml of LB containing Ca^{2+}, and grown at 37°C with shaking at 150 rpm to an OD_{600} of 0.3-0.4. At this time, 2 x 10^{-3} KCN and P1 heavy-light lysate were added at a multiplicity of infection of 2, and

then incubation was continued for 20min at 37°C with shaking at 150 rpm to allow P1 to be absorbed. Bacteria were spun down at 5000g for 6min and then resuspended in 200 ml of fresh LB without Ca^{2+} and in the presence of 0.1 M PSMF as proteinase inhibitor, then grown at 37°C until lysis. Chloroform was then added and bacteria were grown at 37°C for a further 15min to complete lysis. Cell debris including the chromosomal DNA was removed by centrifugation at 30,000 g in a SS-34 rotor for 15min at 4°C. Supernatant, which contains abortively transduced DNA, P1 particles, and remaining cell debris including sheared chromosomal DNA, was concentrated using Millipore centricon plus-80 to a total volume of 20 ml. The concentrate was dialyzed against ExoV storage buffer (20 mM Tris-HCl, pH7.5; 1 mM 2-mercaptoethanol; 0.1 mM EDTA), for at least 3 changes. 80 µl of Exonuclease V (0.2µg/µl) with 20 mM Mg^{2+}; 0.2 mM ATP; 10x reaction buffer (pH7.4) was added and incubation was carried out at 37°C for 30min. Additional ATP was added at 30min, 60min and 140min for a total 180min process to remove linear DNA. An aliquot was taken at the end and an additional 0.6 mM ATP added and left overnight at 37°C as a control. RNase was added to a final concentration of 100 µg/ml for 1 hour at room temperature. The samples were then loaded onto a CsCl gradient, consisting of 1.5 g/cm^3 CsCl solution and a 1.8 g/cm^3 FC-40 cushion, and centrifuged at 27k rpm, for at least 30 hours at 25°C in a TH641 rotor. DNA including abortively transduced DNA, sheared chromosomal DNA and other DNA was drawn from the interface of the two phases, and then dialyzed versus the storage buffer of ExoV for 3-4 changes.

2.4. DNA techniques

2.4.1. Crossover PCR deletions and subcloning

The procedure is a modification of the method described by Church's lab (Link *et al.*, 1997). Crossover PCR products were constructed in two steps. In the first step, two different PCRs were used to generate fragments to the right and left of the sequences targeted for the deletion. Within these two PCR products, two overlapping tails, which contain the desired cloning sites, were introduced in order to produce a fusion product later on. In the second step, the left and right fragments were annealed at their overlapping region and amplified by PCR as a single fragment, using the outer primers in the first step. The fusion product was then digested by desired restriction enzymes, gel purified and ligated into the target plasmid (see Fig. 2.1) The following conditions were used to carry out PCR: "hot start" at 95°C for 5min without polymerase; denaturing at 95°C for 30sec; annealing at 55°C for 1min and extension at 72°C for 1.5min. If needed, a final 5min 72°C hold step was applied and all experiments used 30 cycles. The primers used to carry out gene replacements and deletions are listed below (Table 2.5.) and all working concentration of primers is 30 μM.

Table 2.5. List of the primers for gene replacements and deletions

Primer name	5'- Primer sequence - 3'	Description
PCl	cagctgggatccctgaaaccgttgattgatgcc	argC left arm
PCr	ggagctctaaattgattctcaatttggccattaaataagagactgcgt	argC right arm
PHC	tggccaaattgagaatcaatttagagctccagagttatggcactttgg	argH left arm
PHr	cgttagggatccaacataggccaggcaccagtg	argH right arm
phoR-5	tcgaaaggatccgccgcaccgacg	phoR left arm
phoR-3	tgactggagctcaaataagccagc	phoR right arm
brnQ-5	gctgtgcggccgccagggattggc	brnQ left arm
brnQ-3	ctcagtgtcgacgctgatacaacc	brnQ right arm
35dsdA-5	aaaaaggatccaatttgcccagcaaggccgtatcg	dsdA left arm
35dsdA-3	aaaaagagctcacgttattaacggccttttgccag	dsdA right arm
emrY-5r	cgcagcgctagcatcctttccctg	emrY left arm
emrY-3r	gtatgggcgcgcctattactggcg	emrY right arm
tyrR-5r	tttgcgggatccagaagaatctgg	tyrR left arm
tyrR-3r	aggcatgagctcgcttactcttcg	tyrR right arm
tpX-5r	cgaatagcggccgcggtgcaacac	tpX left arm
tpX-3r	tccagggtcgaccggttacagtcg	tpX right arm
dacB-5	acggtcggatccagtaaacagtcg	dacB left arm
dacB-3	gtttctgagctcctaattgttctg	dacB right arm
35yhbZ-5	aaaaagcggccgcgcttcagattattgacaaagtg	yhbZ left arm
35yhbZ-3	aaaaagtcgaccgttgccgcgtcctgttgcacctc	yhbZ right arm

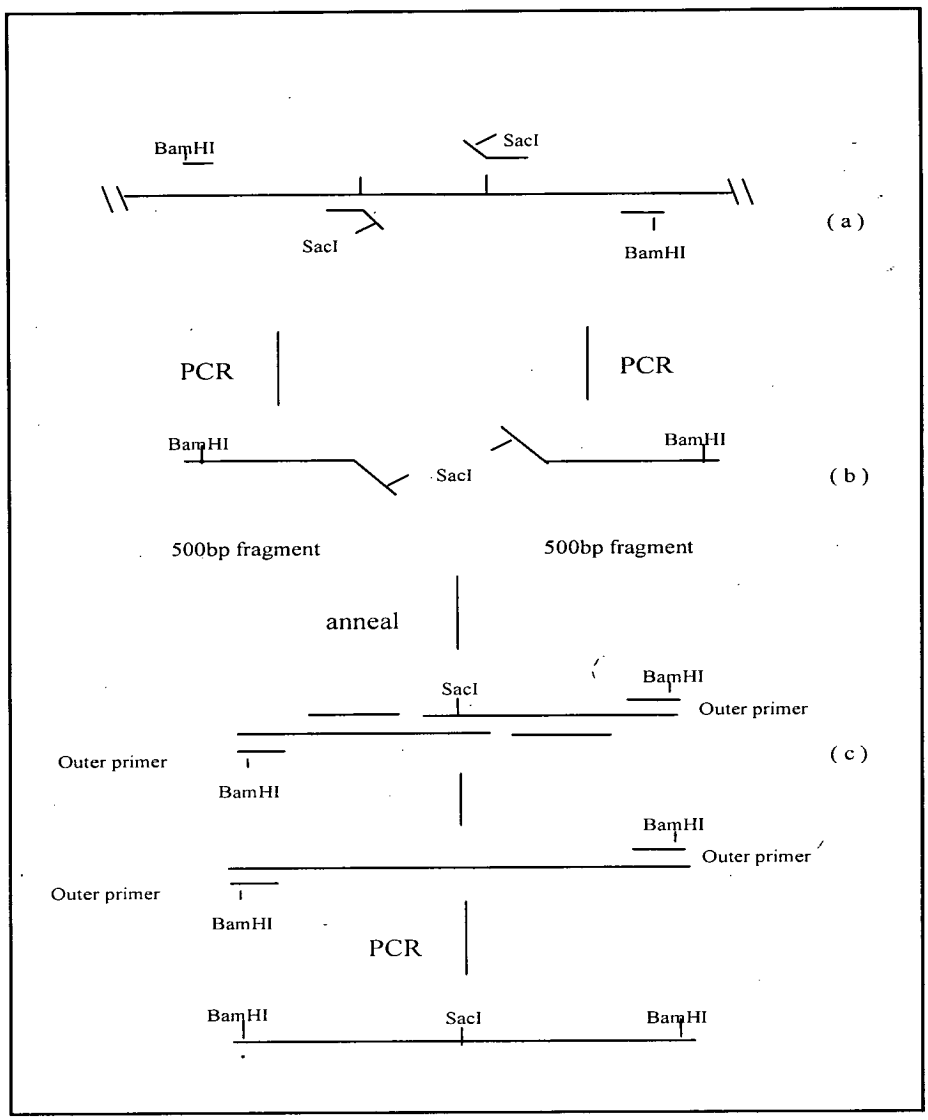


Fig 2.1 Crossover PCR technique

2.4.2. DNA digestion and ligation

A total volume of 420 µl of plasmid was prepared using Promega SV mini prep. Kit. 250 µl of the above extraction was mixed with 100 U of each restriction endonuclease and 10x buffer, then incubated at 37°C overnight. The digested vector was purified and concentrated to a total volume of 30 µl with Qiagen PCR purification kit and was then ready to ligate with insert. PCR products were digested with the same restriction endonucleases as above, run on an agarose gel and then purified with Qiagen gel extraction kit. 4 µl of both vector and insert were mixed with 10x ligation buffer and T_4 ligase and incubated at 16°C for at least 16 hours.

2.4.3. Extraction of P1 DNA

A P1 lysate (plate or liquid) was made and chloroform and cell debris were removed. 10 µg/ml of DNase I and RNase A were then added and incubation was continued at room temperature for 1 hour. (For large scale liquid lysate: phages were then concentrated with PEG precipitation procedure as follows: 40 g/L of NaCl and 10% of PEG8000 were added into the P1 liquid lysate and stirred to dissolve, and then the liquid was left at 4°C overnight. Phages were collected by centrifugation with a GSA rotor at 13K rpm for 30min and resuspended in a small volume of phage buffer using a rotary shaker at 4°C overnight.) Phages were loaded onto a CsCl step gradient, consisting of 2.5ml of 1.3g/ml; 2ml of 1.5g/ml and 1.5ml of 1.7g/ml CsCl solutions and then centrifuged with a TH641 rotor at 35k rpm for 35min at 20°C. The phage band was pulled out and dialysed against phage buffer (500ml/change, 4 changes) at 4°C overnight. 50 µg/ml of DNase I was then added and incubation was carried out at room temperature for 1 hour. Phages were banded again on a 1.5 g/ml CsCl equilibrium gradient with a Ti50 rotor at 45k rpm overnight (or at 38k rpm for 48-72 hours). The phage band was pulled out and dialysed against phage buffer again as described above. 50 µg/ml of proteinase K was added and incubation was carried out at 65°C for 1 hour. P1 DNA was extracted by phenol extraction (3 times), $CHCl_3$/IAA extraction and diethyl ether extraction (3 times). Ether was removed by speed vacuum at high mode for 10min and then P1 DNA was dialysed against 1 x TE buffer (500ml/change, 4 changes) at 4°C overnight.

2.4.4. Slot blotting

The DNA samples were heated in a total volume of 0.5 ml with a final concentration equal to 0.4 M NaOH, 10 mM EDTA at 100°C for 10min. A sheet of Zeta-probe GT membrane was wetted by immersing it in distilled water. The microfiltration (PR648 slot-blot manifold) apparatus was then assembled with pre-wetted membrane. Wells were rinsed with 0.5 ml of TE or H_2O, and then vacuum was applied until wells were empty but not dry. 0.5 ml of the DNA samples were applied into appropriate wells without vacuum and afterwards, vacuum was applied until the wells were just dry. All wells were rinsed by placing 0.5 ml of 0.4 M NaOH in each, and then vacuum was applied again until all wells were quite dry. When the vacuum had been disconnected and the apparatus disassembled, the membrane was briefly rinsed in 2 x SSC and air dried. The DNA was finally cross linked on the membrane under UV irradiation. The membrane was then ready to be hybridized by either DNA probe or antibody against DNA.

2.4.5. Southern blotting

P1 DNA was purified from P1 lysates and then digested with EcoRV for more than 4 hours. The digested P1 DNA along with a series of concentrations of EcoRV-treated chromosomal DNA was fractionated by running on a 0.8% agarose gel. The gel was run at 30V overnight to ensure that the bands were separated well and then stained and photographed the next day.

Southern blotting was carried out in two steps. In the first step, the digested DNA was transferred from an agarose gel onto the membrane. In the second step, DNA was hybridized with DNA probes.

Step1: DNA Capillary Transfer – DNA was depurinated by soaking the gel in 0.25 M HCl for 10-15 minutes if the DNA fragments were bigger than 4kb. 4 sheets of probing paper was cut so that they overhung the bottom of the gel tray by 5 cm on each end and at the same time Zeta-Probe GT membrane was pre-wetted in distilled water. The four sheets of the paper were placed on a tray and then the tray was placed with the paper in the bottom of a deep dish. The paper was saturated with 0.4

M NaOH. Bubbles were removed and enough NaOH was poured into the deep dish so that the ends of the probing paper were immersed in NaOH, then the gel was carefully placed onto the saturated filter paper. The gel was checked carefully to ensure that no bubbles were trapped beneath the gel. The gel was covered with a small amount of NaOH. Saran wrap was placed over the entire gel/paper stack and a window cut out, allowing only the gel to emerge. The sheet of pre-wetted membrane was slowly lowered onto the gel surface and the filter surface was carefully flooded with NaOH. Again, it was made sure that no bubbles were present between gel and membrane. Two pieces of probing paper were cut exactly to the gel size, wetted and placed onto the membrane/gel stack. Any bubbles were removed from beneath each layer of filter paper. A stack of paper towels was placed on the paper/gel/membrane stack. The paper towel stack was covered with a glass plate and the pressure on the paper towel stack was kept at a minimum. The transferring was continued for at least 16 hours, depending on the gel concentration and fragment size. After transfer, the membrane was separated from the gel, rinsed briefly in 2x SSC, and air dried.

Alternatively, DNA was depurinated by soaking the gel in 0.25 M HCl for 10-15 minutes if the DNA fragments were bigger than 4kb and then directly denatured in 0.5M NaOH - 1.5M NaCl solution for 1 hour and neutralized in 1M Tris-HCl pH 8.0 - 1.5M NaCl solution for 1 hour, and then placed on 2 sheets of filter paper pre-wetted in distilled water. Other steps were performed as described above, except no NaOH was required.

Step2: after transferring the DNA from a gel to the membrane, the membrane was dried and the DNA was fixed on the membrane by cross linking with UV. Membrane was placed into a hybridization bottle with 20 ml of hybridization solution and then incubated at 65°C, rolling at least for 1 hour. During the period of pre-hybridization, probes were prepared by two steps: first, the desired genes were PCR amplified and purified. Second, the probes were made by labelling with High prime kit (Roche) by simply following the protocol coming with the kit, and then Amersham Nick kit was used to remove the background. Purified labelled probes were then denatured again by heating at 100°C for 5 min, cooled down to the incubation temperature (60°C in this work) and mixed with the pre-hybridized membrane in the bottle. Hybridization was carried on overnight. Overnight hybridization solution was kept in a universal bottle and stored in the freezer (optional) the next day. The membrane was washed 3 times at 55°C (10°C lower than the hybridization temperature) for 15 min with 20 ml of

fresh hybridization solution each time. The membrane was wrapped up with Saran film tightly and then after all bubbles were removed, put into an autoradiography cassette to allow to autoradiograph for 1-2 days, depending on how strong the signal was. The hybridization bottle was washed thoroughly.

DNA concentration was calculated by measuring OD_{260} with a UV spectrophotometer. The formula used to calculate DNA concentration is as follows: OD_{260} x dilution x 50 = μg/ml.

2.4.6. Western-slot blotting for detecting DNA

Membrane was prepared as described for slot blotting, blocked in 5% non-fat dried milk in PBS/T (0.1% Tween20 in PBS) for 60 min at room temperature on an orbital shaker and then rinsed with PBS/T for 2 min twice. The membrane was incubated with primary antibody in PBS/T/5% milk for 60min at room temperature on the orbital shaker, then rinsed with 2 changes of PBS/T and followed by washing 15 min at room temperature. The membrane was washed 3 more times for 5 min each at room temperature, then incubated with secondary antibody in PBS/T for 60 min at room temperature on the orbital shaker and then washed as above. Amersham ECL Plus was used to produce the signal as follows: mixed detection solutions A and B in a ratio of 40:1 and the final volume of detection reagent required is 0.1 ml/cm^2. Excess liquid was drained from the membrane and placed side up on Saran Wrap. Mixed detection reagent was added to membrane for 5 min at room temperature. Excess detection reagent was drained off by touching the edge against tissue and placing sample side down on fresh Saran Wrap, and then wrapping up and removing air bubbles. Then the membrane was ready to be exposed to film.

2.5. Gene replacement and deletion techniques

2.5.1. Protocol of integration of a *pac* site onto the chromosome with pKO3-based plasmid

The pKO3-based plasmid was transformed into a recipient strain by either chemical methods or by electroporation as described above. The bacteria were grown at 30°C overnight. On the next day, colonies were picked up from the overnight plate and streaked out on a LB plate containing Chloramphenicol and Kanamycin, and then incubated at 43°C overnight. Several big colonies from different parents on the overnight plate were re-streaked out on a new LB (Cmp^+, Kan^+) plate the next day, then incubated at 43°C overnight. Several big colonies from different parents on the above plate were picked up and put into 5 ml of LB in a Bijou, then incubated at 30°C overnight. The overnight culture was then serially diluted 10, 100 and 1000-fold and spread onto LB containing 5% sucrose and Kanamycin and then incubated at 30°C overnight. Single colonies from sucrose plates were picked up and patched out on an LB plate containing 5% sucrose and Kanamycin and an LB (Cmp^+, Kan^+) plate separately at the same time, then placed at 30°C overnight. Cells which could grow on the LB containing 5% sucrose and Kanamycin but not the LB (Cmp^+, Kan^+) plate, were the strain which had the *pac* site integrated on the chromosome. PCR was used later to verify the results.

2.5.2. Protocol of FRT specific recombination

(to remove KAN cassette from the chromosome)

An Flp-containing plasmid (temperature sensitive, Ampicillin resistant) was transformed into *pac*-KAN cassette inserted cells. The bacteria were then spread on an LB plate containing Ampicillin and grown at 30°C overnight. Ampicillin resistant colonies were selected from the plate on the next day, and then purified once by streaking out a single colony on an LB plate containing Ampicillin and incubating at 30°C overnight. A single colony from the above overnight plate was picked up and

streaked on a KAN plate to check if the KAN cassette had been lost. At the same time, another single colony from the same overnight plate was streaked out on a LB plate and grown at 42°C overnight to cure the plasmid. A single colony was picked up the next day from the overnight plate and purified once by streaking out on a fresh LB plate and incubated at 42°C overnight. The plasmid-cured cells were then checked by streaking a single colony from the above overnight plate on a LB plate containing Ampicillin and grown at 30°C overnight. The final strain should be Ampicillin resistant and Kanamycin sensitive.

2.6 Extraction of DNA from P1 – infected cells

This method is a modification of the method described by Sternberg and Coulby (1987). The overnight cultures were diluted 50-fold into LB Ca^{2+} and grown to an OD_{600} of about 0.4 (about 1 x 10^8 cells). Phage P1, following addition of 2 x 10^{-3} M KCN^-, was added into the cultures with a m.o.i. of 4 and incubation was continued at 37°C for 15min with shaking. P1 infected cells were then pelleted and washed with fresh pre-warmed LB once and resuspended (diluted 5-fold) in fresh LB (without Ca^{2+}) and grown at 37°C.

5 ml samples of P1 – infected cells were taken and chilled to 4°C (on ice) at various times (usually every 10min) from the beginning after resuspension and labelled MG0'(5pac0') – the aliquot of P1-infected MG1655 (or MG1655(5pac)) was taken right after washing and resuspension; MG10' (5pac10') – the aliquot of P1-infected MG1655 (or MG1655(5pac)) was taken 10min later after resuspension and so on. The aliquots were immediately resuspended in TELS buffer and then centrifuged at 7k rpm for 5min in an SS34 rotor. The pellets were resuspended in 1ml of 50mM Tris-HCl (pH8.0), 10% (w/v) sucrose. Lysozyme was added to 1mg/ml and EDTA to 20mM and cells were incubated for 15min at 4°C. SDS was then added to 0.1% (w/v) to lyse the cells, followed by adding RNase A (20μg/ml) to the lysate and incubating at 30°C for 20min. Proteinase K was then added to 100μg/ml and incubation was continued for 60min at 37°C.

DNA was extracted twice using phase lock gels (Eppendorf® product) with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, v/v) and twice with chloroform/isoamyl alcohol (24:1, v/v). The extracted DNA was then precipitated with 1 – 1.5 volume of isopropanol and spun down at 16k g for 15min using a desktop centrifuge. The pellets were washed with 1ml of 70% ethanol and re-centrifuged at 16k g for 5min using a desktop centrifuge. DNA was resuspended in 200μl of water (or TE) overnight at room temperature and stored at 4°C.

Chapter III

Purification and detection of abortively transduced DNA and protein

3.1. Introduction

In this project, I have been trying to investigate the nature of abortively transduced protein (AT-protein) in order to answer the following questions: is AT-protein encoded by the host or by P1 DNA? What is the normal function of the protein? How is the protein involved in the formation of abortive transductants?

Most transduced DNA, up to 90%, persists in the recipients in a form refractory to degradation. This nonchromosomally associated DNA [designated abortively transduced DNA (Sandri and Berger, 1980a)] is not replicated although it can be transcribed (Lederberg, J., 1956) and is stable. The mechanism of formation of abortively transduced DNA (AT-DNA) is not clear yet. But it is believed that a protein, the so-called abortively transduced protein, plays an essential role in the formation of AT-DNA by joining the ends of the transduced DNA to render it circular. This hypothesis is supported by the following observations. Ikeda and Tomizawa (1965b) found that a protein is specifically attached to the transducing DNA, but not the infective DNA. Later, Sandri and Berger (1980b) demonstrated that abortively transduced DNA is circular with a protein associating with the two ends.

Identifying this protein, although intriguing, has proved to be technically difficult, and I was ultimately unsuccessful. In this chapter I will describe the methods I used and the results obtained. What I tried to do is to purify sufficient AT-DNA to provide an adequate source of AT-protein. Some new approaches I designed for this purpose and the progress I made is described in this chapter as well as the difficulties that arose.

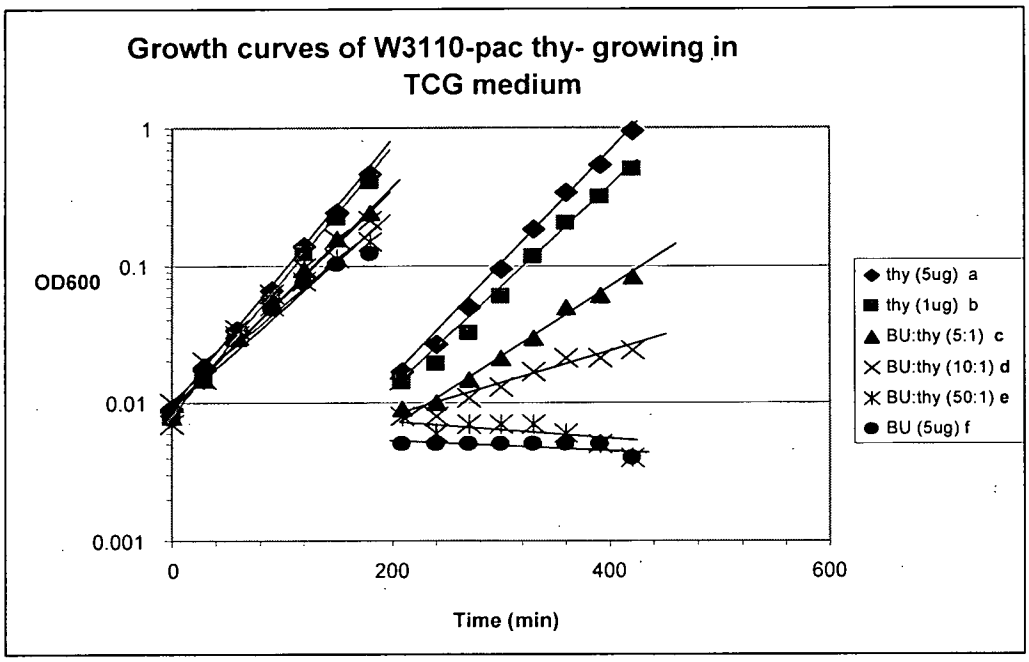
3.2. BU labelling P1 transducing particles

Abortively transduced DNA consists of bacterial chromosomal DNA transduced by P1 transducing particles. Therefore, ideally, to study abortively transduced DNA it is necessary to separate the transducing particles from the much larger number of infective particles present in lysates. Once purified, the transducing particles can be used to infect a recipient cell to produce abortively transduced DNA in the absence of P1 infection. So, although it is impossible to separate transducing particles from infectious particles completely, since less than 1% of P1 phages are transducing particles, the higher the purity we can attain, the better for the subsequent experiments.

As mentioned above, the only difference between transducing particles and infective particles is the type of DNA encapsidated. The former is exclusively chromosomal DNA packaged from host cells and the latter is P1 genomic DNA made after infection. Since all the characteristics of these two types of particles are nearly the same, the only way to separate them relies on the difference in buoyant density caused by the two different types of encapsidated DNA. Because transducing DNA is host DNA made principally before infection, it is possible to label it with a density label before infection. 5'-Bromouracil (BU) is a higher-density analog of thymine which can be incorporated into chromosomal DNA in place of thymine and it was used here to increase the tiny density difference between these two types of particles, which is about $0.002 g.cm^{-3}$ without labelling (Ikeda and Tomizawa, 1965a).

The labelling protocol is modified from Sandri and Berger's and described above in the chapter of methods and materials. Thymine-requiring cells were grown in a medium containing 5 parts BU to 1 of thymine for several hours. This ratio is optimized to ensure that cells can grow normally, since without any thymine in the mixture, cells will grow only slowly (Fig 3.1). It is critical in this experiment to label only the chromosomal DNA with BU. To achieve this result, the following steps were employed: 1) Ten minutes before adding P1 to the culture, the concentration of thymine was increased to 50μg/ml, 10-fold higher than that of BU, in order to dilute the concentration of BU. 2) After phage adsorption, cells were centrifuged and resuspended to ensure that BU was completely removed from the culture. Anti-BrdU antibody was used in the experiment shown in Fig 3.2. Cells

grown in BU-containing TCG medium were harvested before P1 was added and their chromosomal DNA was extracted and spotted on to filters. Using anti-BrdU antibody, we can clearly see that the chromosomal DNA has been labelled by BU. P1 lysates grown under different conditions were also measured by dot blot (Fig 3.3). Samples of lysates in which BU was present throughout infection, in which BU as only present before infection or in which no BU was used were spotted onto filters. From the result of the dot blot, we can see that the signal intensity for P1(heavy-light) lysates, in which only P1 transducing DNA is labelled, is between that for P1(BU-only) lysates, in which both transducing DNA and P1 genomes were labelled and that for P1(+thy only) lysates, which was used as a negative control. No signal could be observed for P1(+thy only) lysates, while P1 lysates in which BU was present both before and after infection gave a very intense signal much higher than P1(heavy-light) lysates, since P1 infective particles comprise about 99% of the total particles.



A

W3110-*pac* thy⁻	Medium conditions					
	Thy (5μg/ul)	**Thy (1μg/ul)**	**BU : Thy (5:1)**	**BU : Thy (10:1)**	**BU : Thy (50:1)**	**BU (5μg/ul)**
Doubling time (min)	33	35	51	74		

B

Fig 3.1 Growth curves of W3110-*pac* thy⁻ growing in TCG medium in order to test the optimal ratio of BU to thy. Conditions ((**a**) 5μg/ml thy, (**b**) 1μg/ml thy, (**c**) 5μg/ml BU:1μg/ml thy (5:1), (**d**) 5μg/ml BU: 0.5μg/ml thy (10:1), (**e**) 5μg/ml BU: 0.1μg/ml thy (50:1) and (**f**) 5μg/ml BU only) were tested. 5μg/ml BU:1μg/ml thy (5:1) appeared to be the optimal ratio for this purpose, which is consistent with the previous work. **A**) Growth rates of W3110-*pac* thy⁻ growing in different conditions. **B**) A table shows the doubling time of the cell growing in different conditions.

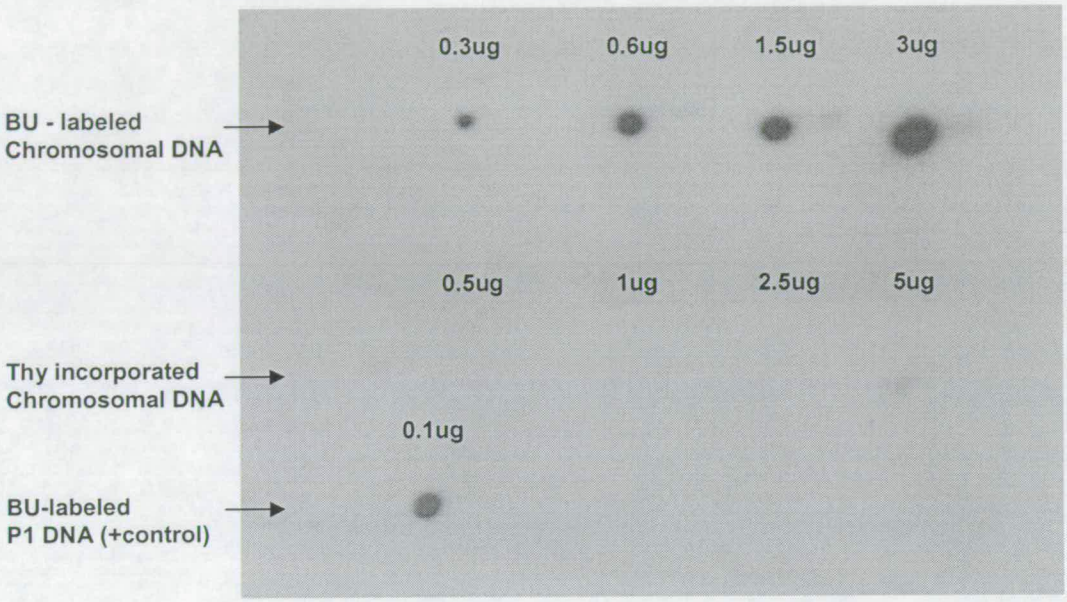


Fig 3.2 Dot blot to detect the BU labelling of chromosomal DNA. Cells were grown in TCG medium with 5 μg/ml BU and 1 μg/ml thy at 37°C for 4 hours as described in Materials and Methods. Before P1 was added, an aliquot of the culture was taken and cells were harvested by centrifugation. The chromosomal DNA was extracted with *Bio-Rad* Genomic DNA Isolation Kit. Purified chromosomal DNA was spotted onto the filter and detected with ECL-plus Western Blotting Detection System (Amersham pharmacia Biotech) using anti-BrdU antibody. thy-incorporated chromosomal DNA and BU-labeled P1 DNA were used as controls.

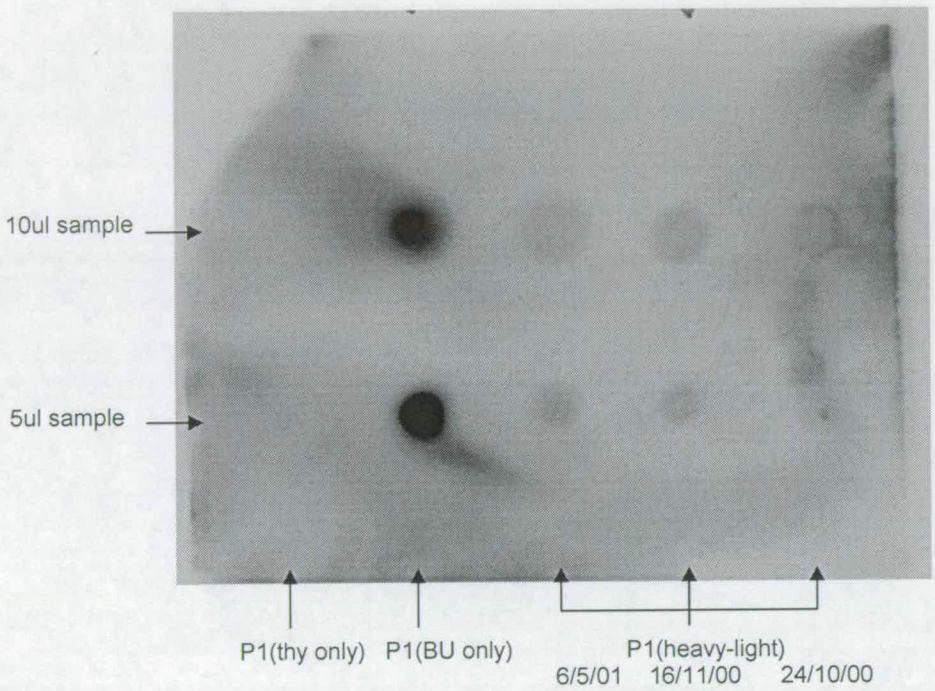


Fig 3.3 Dot blot to detect the labelling effect by BU of the P1 transducing particles. Anti-BrdU antibody was used for this purpose. The procedure is described in Methods and Materials. P1 lysates were directly dropped onto the filter and proteins were denatured and removed on the membrane to release DNA. All lysates have a titer of about 10^{11} pfu/ml. **P1(heavy-light)** stands for P1 lysates in which P1 transducing particles are labelled by BU as described above, but not P1 infective particles. **P1(BU only)** stands for a P1 lysate which was made in presence of BU throughout the procedure, so that all P1 genomes have been labelled by BU. **P1(+thy only)** stands for P1 lysates which was made without supply of BU throughout the procedure.

3.3. Extraction and analysis of abortively transduced DNA

3.3.1 Separation of transducing particles from infectious particles by CsCl equilibrium gradients

In previous work, CsCl equilibrium gradients were often used to separate infectious and transducing P1 particles. The expected densities for BU-labelled P1 transducing particles and T-T infective particles reported by Sandri and Berger, (1980a) are 1.46-1.47g.cm^{-3} depending on the genetic marker and 1.45g.cm^{-3} respectively, while Ikeda and Tomizawa, (1965a) reported 1.48g.cm^{-3} and 1.47g.cm^{-3} respectively. Therefore, the expected difference between these two particles would be 0.01g.cm^{-3}.

Preparation of CsCl equilibrium gradients, centrifugation and fraction collection were carried out as described in Methods and Materials. However, after centrifuging, only one obvious band appeared, suggesting that the transducing particles are either too few to give a visible band or not sufficiently different in density to show a band fully separated from the infective particles. Fractions were collected by piercing the bottom of the tube and analyzed by both titration and transduction and also by western dot-blot with anti-BU Antibody (Fig. 3.2, 3.3). From the dot-blot (Fig 3.4, Table 3.1), we can see an apparent peak in which transducing particles are likely to be located (C5 to D6 fractions), while infectious peak lagged a couple of fractions behind from the titration result. In the experiment shown in Fig 3.5, the peak of transducing particles was located by measuring the number of *trp* transductants obtained from each fraction, while the peak of infectious particles was located according to the titer of each fraction. As we can see, the titer of the fraction which transduces *trp* best is about 10^8, which is consistent with Sandri and Berger's results (1980a). Thus, after centrifugation, two types of P1 particles have been at least partially separated and the fractions containing mainly transducing particles were ready to be used for the subsequent experiments.

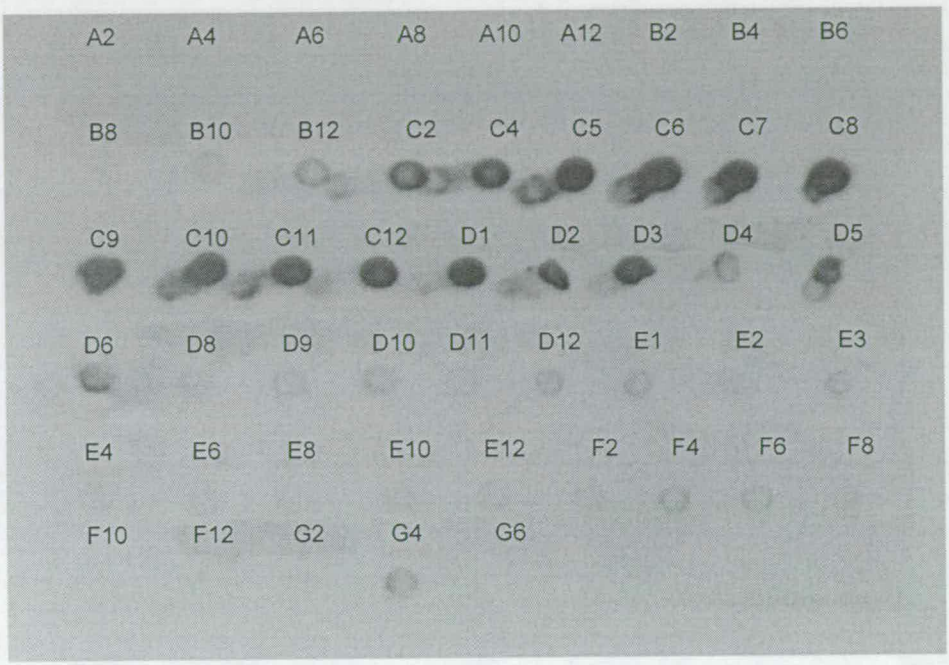


Fig 3.4 Western dot-blot of fractions to detect separation effect of two types P1 particles after centrifugation on CsCl equilibrium gradient (at 21.6 k rpm, 20°C in SW55 Ti for 20 hours). Fractions were collected into a 96-well plate by piercing the bottom of the tube. From A – G is from the bottom to the top of the tube. 78 fractions in total were collected. Each fraction was diluted to 300 µl with phage buffer. Each fraction (P1 particles) was spotted onto a piece of Nylon membrane (positively charged). In order to remove phage capsid, the membrane was soaked in 1% SDS, 0.5M NaOH, 1.5M NaCl on a rotary platform for 1 hour and then followed by neutralization in 1 M Tris (pH = 7.4), 1.5M NaCl for 1 hour. DNA was then cross linked with the membrane by UV. Dot-blot was carried out as described in Materials and Methods.

Fraction No.	Titer (pfu/ml)	Titer(pfu/fract.) 300µl/fraction	Transd. (*trp*) No. on the plates	Transd. No./ fraction	Transd./titer (per fraction)
C2	1.2×10^{6}	3.6×10^{5}	87	2160	6.0×10^{-3}
C4	3×10^{6}	9×10^{5}	161	4830	5.4×10^{-3}
C6	1.6×10^{7}	4.8×10^{6}	272	8160	1.7×10^{-3}
C8	1.5×10^{8}	4.5×10^{7}	454	13620	3.0×10^{-4}
C10	7×10^{8}	2.1×10^{8}	624	18720	8.9×10^{-5}
C12	4×10^{9}	1.2×10^{9}	407	12210	1.0×10^{-5}
D2	1.2×10^{10}	3.6×10^{9}	181	5430	1.5×10^{-6}
D4	3×10^{10}	9×10^{9}	69	2070	2.3×10^{-7}
D6	3×10^{10}	9×10^{9}	50	1500	1.7×10^{-7}
D8	7×10^{10}	2.1×10^{10}	41	1230	2.8×10^{-8}
D10	6×10^{9}	1.8×10^{9}	27	810	4.5×10^{-7}
D12	4×10^{9}	1.2×10^{9}	32	960	8×10^{-7}

Table 3.1 Titration and transduction results of each fraction after separation by CsCl equilibrium gradient. The total number of input phage is 2×10^{11}. Transduction was carried out as follows: 10 µl of each fraction was mixed with 100 µl MM303 (OD_{600} = 1-1.5) Incubation was carried out at 37oC for 15min and then, 2.5 ml top water agar was added into the mixture and poured onto the corresponding *trp*⁻ plate.

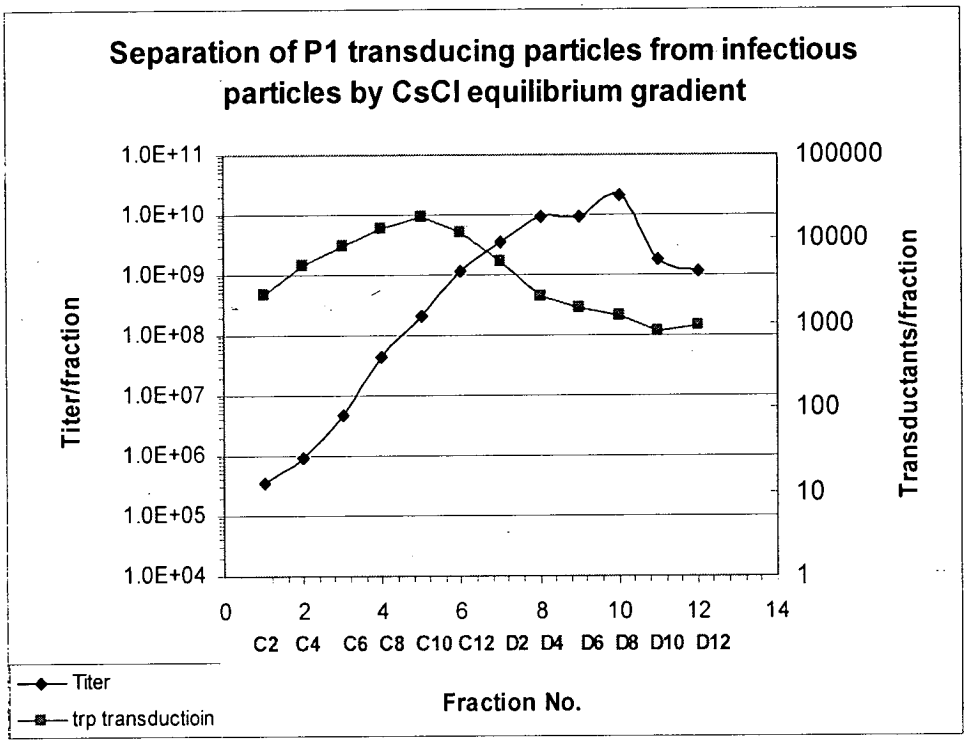


Fig 3.5 CsCl density-gradient centrifugation analyses of transducing particles carrying *trp* marker and infectious particles. Data is summarized in Table 3.1 and the dot-blot result is shown in Fig 3.4

3.3.2 Purification and detection of abortively transduced DNA

I was now ready to use partially purified transducing particles to infect new recipients with the objective of purifying AT-DNA. The fractions which had high transducing activity were mixed together and used to transduce the recipient cells. Then, the cells were lysed with Triton X-100 to release the abortively transduced DNA (AT DNA) and other cellular contents. Most of the cellular contents were removed by centrifugation at 30,000g in an SS-34 rotor for 15min at 4°C (Sandri and Berger, 1980b). In Sandri's work, the AT-DNA which had been labelled by ^{32}P was collected by centrifugation on CsCl equilibrium gradients, where it could be identified as constituting radioactive fractions. However, I could not use this approach, as the transducing DNA in my experiment was not radioactively labelled (discussed later). I therefore set up a step gradient, consisting of a layer of 4 ml of 1.5g.cm^{-3} CsCl and a cushion of 2 ml of Fluorinert FC40 (Sigma, 1.85g/ml) to allow me to collect all DNA at the interface after centrifugation for more than 30 hours (Keeney *et al.* 1997, Cell 88), since the density of DNA is about 1.7g.cm^{-3}. Slot blot with anti-BrdU antibody was used to detect the abortively transduced DNA in the total withdrawn DNA (Fig 3.6).

We can see a faint band appearing on the filter for the P1 AT-DNA sample in Fig 3.4, while no signal could be seen for P1 DNA (-control) and a rather strong signal for P1 (BU) DNA (+control), indicating that some BU-labelled DNA was recovered from the recipient cell. Considering the procedure for lysis of cells and the fact that 90% of transduced DNA in the recipient cell forms abortively transduced DNA, this band might be derived from abortively transduced DNA, though we could not completely rule out that the result is contaminated by some BU-labelled P1 DNA from donors. However, since the amount of phage used in the transduction was large and the signal obtained only faint, the amount of AT-DNA obtained by the above procedures was not enough to do further analysis on AT DNA and therefore on AT protein.

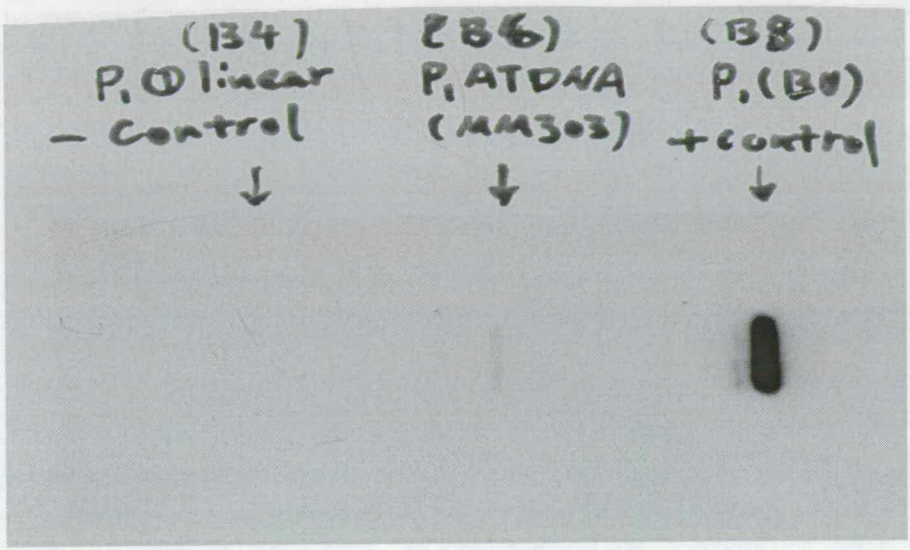


Fig 3.6 Western slot-blot to analyze BU-labelled abortively transduced DNA. P1(BU) stands for BU-labelled P1 DNA. P1 linear (-control) is P1 DNA without labelling. P1 AT-DNA is the DNA withdrawn from the interface of the step gradient. BU-labelled P1 DNA shows a rather dark band, while normal P1 DNA (- control) shows no signal, indicating that the anti-BrdU antibody was working properly. A faint band appears in the slot of DNA extracted from recipient cells, suggesting that some BU-labelled DNA was recovered from the recipient cell and supposed to be AT-DNA.

3.3.3 Discussion on separation of two P1 particles using CsCl gradient

Summary on BU-labelling approach

BU-labelling of chromosomal DNA followed by separation of DNAs by density on CsCl gradients was a widely used method to separate materials with very similar buoyant densities in the past. The key for successful separation is that BU can only be incorporated into the host chromosome, but not into the P1 genome. The method used in this work was modified from previous work by Ikeda and particularly, Sandri and Berger.

Although many steps were involved to ensure the specificity of BU incorporation as described above and the substitution of bromouracil by thymine was supposed to be rapid and complete (Ikeda and Tomizawa, 1965a), we cannot completely rule out the possibility that some P1 genomes may have incorporated BU as well. But fortunately, according to the dot-blotting results (Fig. 3.2), we could conclude that the host chromosome had been labelled by BU with quite high specificity. Even though there might be some P1 genomes which had been labelled too, the amount of these genomes should be rather low and could possibly be able to be ignored as compared to the result of P1 (BU) positive controls in Fig 3.3.

One might also ask whether it could be possible that the signals of P1 (heavy-light) lysates in Fig 3.3 were derived mainly from BU labelled P1 genomes, considering the fact that P1 infectious particles comprise about 99% of the lysate. However, this could not be true, because the fractions showing the strongest signals after western dot-blot were not the fractions having the highest titer after centrifugation on CsCl equilibrium gradient (Fig 3.4).

Summary on separation by CsCl equilibrium gradient

Separation on CsCl equilibrium gradients is another important step. As a matter of fact, I spent most of my time working on finding suitable conditions to produce the best separation. In Sandri's

work, they tended to use short centrifugation time (4hr) with a relatively high speed (149,000 g) to avoid losing P1's viability as a result of prolonged exposure of P1 to CsCl. However, in my work, I could not find any substantial losses in viability caused by CsCl at least within 2 days and in fact, previous experiments had reported that centrifugation at a high speed for a short time results in a sharp gradient and thus reduces the resolution. To avoid a sharp gradient, I tested several conditions reported in different papers and according to the results, I decided to carry out all my centrifugation work later in the SW55 rotor at 21.6k rpm at 20°C for 20 hours.

To obtain a better separation, I also avoided using PEG8000/NaCl to concentrate P1 particles from a large scale liquid lysate, which I often used to concentrate P1 particles in order to purify P1 DNA, and started to use 3-hour centrifugation without PEG and salt to concentrate P1 particles as described in Sandri's work instead. This is because PEG8000, as an efficient reagent to aggregate P1 particles, may interfere with separation of two types of P1 particles. As a matter of fact, this step appeared to be useful to improve the separation results relative to the results I got at the beginning of this work.

Transduction, titration as well as dot-blot were applied to locate P1 transducing particles and infectious particle bands. Although these methods were laborious, they clearly revealed that separation (Fig 3.5) has been obtained.

Summary on extraction of AT-DNA from recipient cells

As described above, infection of the recipient cell was carried out to produce abortively transduced DNA after transducing particles were partially purified from infective particles. Sandri and Berger, 1980b, concentrated and purified the AT-DNA by banding on a CsCl equilibrium gradient and then analysing by autoradiography directly following agarose gel electrophoresis.

Since we could not use CsCl equilibrium gradients, and instead, the total DNA released from cells was collected using a step gradient, the procedure may not be efficient to separate AT-DNA from other DNA from cells. Although we could manage to detect BU-labelled DNA from the recipient, further work is required to confirm that this DNA is the AT-DNA. However, unfortunately, this result has not been repeated successfully.

In addition, one could imagine that it would be more difficult to analyze AT-DNA on a membrane after transferring this sized DNA onto the membrane following agarose gel electrophoresis as presented in Sandri and Berger's work (1980b). The above results suggest that the procedure that I used above was not efficient and sensitive enough to produce adequate AT-DNA to be analyzed. Therefore, a new method was needed to be developed to achieve the goal.

Technical difficulties encountered in the project

As mentioned above, there were serious technical problems met in trying to purify AT-DNA and in consequence, an AT-protein. Since the gene encoding AT-protein is unknown, one cannot use molecular cloning methods, like over-expression of the encoding gene, to increase the amount of this protein.

Another technical difficulty is that the amount of abortively transduced protein is too low to be studied even by isotope labelling. Since the abortively transduced DNA cannot replicate itself and only derives from transducing particles, the amount of AT DNA is also very low. "The main obstacle lies in the difficulty of preparing large amounts of transducing particles" said Mark C. Hanks in his thesis (Hanks, 1986, thesis). He also commented that the procedure used by Sandri and Berger (1980a) is "technically difficult and yields of abortively transduced DNA are very poor". According to his calculation, the maximum expected yield of abortively transduced DNA from 10^{10} transducing particles, which is the total yield produced by Sandri and Berger, is only 155ng. Considering that the phage strain used by Sandri and Berger is a high-frequency transducing strain P1607H (Wall and Harriman, 1974), which transduces various markers at 5- to 10-fold higher frequencies than the P1vir that Ikeda and Tomizawa used (Sandri and Berger, 1980a), the P1 strain, P1kc, used in this work, which has a similar transduction frequency to the P1vir that Ikeda and Tomizawa used, will yield less abortive DNA. Furthermore, considering that AT-protein, which was estimated at about $5x10^5$ daltons, is only about 0.8% of the amount of the DNA encapsidated in a transducing particle, Ikeda and Tomizawa have reported in their paper (Ikeda and Tomizawa, 1965b) that "separation of the protein from the DNA cannot be studied by labelling the protein with radioactive isotopes ". Apparently, to characterize AT-DNA and AT protein, we have to find a way to increase the amounts of both. Since AT-DNA is derived from transducing DNA, I tried to increase the amount of transducing DNA by

increasing the amount of chromosomal DNA packaged. My approach was to insert the P1 *pac* site into the chromosome in order to increase the amount of packaged transducing DNA. This method definitely increases the numbers of transducing phage carrying markers to one side of *pac*-, but may not increase the total number of transducing phages produced from the entire chromosome (details will be discussed in chapter IV – packaging of multi-*pac* strains).

In the previous work on AT-DNA carried out by Ikeda (Ikeda and Tomizawa, 1965a,b) and Sandri (Sandri and Berger, 1980a-b), the host chromosome was not only labelled by BU, but also by ^{32}P. Measuring radioactivity is a more convenient method than titration and transduction for locating the fractions containing the transducing particles in CsCl equilibrium gradients. It is also possibly more effective than using a step gradient with a Fluorinert FC40 cushion for collecting abortively transduced DNA. It is also more sensitive for detecting and analysing AT-DNA by autoradiography, following agarose gel electrophoresis. However, since the price of ^{32}P proved unexpectedly prohibitive I was unable to use ^{32}P to label the chromosome and used anti-BrdU antibody to detect transducing DNA instead. Therefore, considering these difficulties encountered in the above procedure, a new method was developed to achieve the goal.

3.4 Extraction of abortively transduced DNA using Exo V – a new method

Because AT- protein is easily denatured and removed from the DNA, it is difficult to develop a new approach to purify AT-DNA. In addition, in order to obtain as much abortively transduced DNA as possible, any new method has to reduce purification and transduction steps, because, as calculated in previous work (Sandri and Berger, 1980b; Hanks, thesis, 1986), only 25-40% of transducing particles are able to adsorb to the recipient cells and only 40-60% of these could be re-extracted as abortively transduced DNA afterwards. Thus, the requirements for a new approach are 1) that the method will not destroy the protein attached to abortive DNA; 2) that the method will not degrade abortive DNA; 3) that as few purification and transduction steps as possible should be required. Based on these requirements, a new method was developed by taking advantage of Exonuclease V – recBCD (Table 3.2).

The reason why Exonuclease V (ExoV) was used in this work is because it hydrolyzes nucleotides from both the 3' and 5'- ends of linear double-stranded and single-stranded DNAs, but no nuclease activity is observed on closed circular duplex DNA; other exonucleases either cannot act on both single stranded and double-stranded DNAs or create gaps in DNA (see Table 3.3). The rationale of this procedure is that Exonuclease V (ExoV) will digest linear DNA but that abortively transduced DNA will survive because its ends are protected.

Firstly, the recipient cells were infected by BU labelled P1 (heavy→light) lysates without separation on the CsCl equilibrium gradient and grown at 37°C until the cells lysed. Cells were then centrifuged to remove cell debris. Sheared chromosomal DNA should also be removed at this step (Sandri and Berger, 1980a). Other released cell contents, including some P1 DNA and abortively transduced DNA, and intact P1 phages remain in the supernatant. Secondly, Millipore centricon plus-80 was used to remove water and concentrate the original large volume of liquid to a small volume. Thirdly, ExoV followed by RNase was added to remove RNA and any linear DNA. Finally, the remaining DNA was purified by running on an FC-40 CsCl step gradient as described above, and then

dialyzed versus Hepes buffer and stored at 4 °C. Protein, including intact P1 particles would be expected to float. To detect and analyse abortively transduced DNA, anti-BrdU antibody was used (see Table 3.2).

Table 3.2 Procedure of extraction of AT-DNA. AT-DNA could then be detected by dot or Southern-western blot using Anti-BrdU antibody.

Steps	Purposes
1) Make P1 (heavy→light) lysates	Produce BU-labelled P1 transducing DNA
2) P1 (heavy→light) infects recipient cells	Produce AT-DNA
3) Infected cells are grown to lysis with 0.1M PMSF. $CHCl_3$ was added to complete lysis.	Release AT-DNA and other cellular contents
4) Centrifuge at 30,000g in an SS-34 rotor for 15min at 4°C	Remove chromosomal DNA and cell debris
5) Centrifuge the supernatant from step 4 with Millipore Centricon plus 80	Concentrate the big volume of liquid to a small volume
6) Dialyze the concentrated supernatant from step 5 against ExoV storage buffer	Prepare for step 7
7) ExoV along with ATP is added and reaction is carried out for 3 hours at 37°C.	Remove linear DNA
8) RNase is added to a final concentration of 100µl/ml for 1 hour at room temperature	Remove RNA
9) The concentrated samples were loaded onto a CsCl step gradient and centrifuged at 27k rpm, for at least 30hrs at 25°C in a TH641 rotor	Separate DNA from other cellular contents, such as proteins and P1 phage. DNA is at the interface of the two phases.
10) Dialyze DNA from CsCl gradient against ExoV storage buffer	Prepare for step 11
11) Repeat step 7 if necessary	Remove remaining linear DNA
12) Phenol extraction as described in other DNA extraction procedures	Remove enzymes

Table 3.3 Functions of some well-known exonucleases. (from Amersham Biosciences catalogue 2003)

Name of the enzyme	Functions
Exonuclease I	Acts specifically on single-stranded DNA degrading it processively in the 3'- to 5'- direction producing 5'-mononucleotides
Exonuclease III	Activities including double-stranded specific 3'-5'-exonuclease, DNA 3'-phosphatase and endonucleases at apurinic sites in DNA
Exonuclease V	Hydrolyzes nucleotides from 3'- and 5'- ends of linear double-stranded DNA and single-stranded DNA
Exonuclease VII	A strict single-stranded directed enzyme with 5'-3' and 3'-5' exonuclease activities.

3.4.1 Optimizing the working conditions for Exonuclease V

As described in Chapter 1, ExoV is not only an ATP-stimulated nuclease, but also an ATP-dependent helicase. Many factors, such as the concentrations of Mg^{2+} and ATP can affect or even reverse the activities of the enzyme. High concentrations of ATP increase ds DNA unwinding relative to DNA degradation, while all nuclease activities are inhibited in the presence of Ca^{2+} (Muskavitch and Linn, 1982). Moreover, when RecBCD encounters a recombinational hot spot, called chi (χ), the 3'-5' exonuclease activity on double-stranded DNA is suppressed (Yu *et al.*, 1998). Chi is a common 8 bp sequence (5'-GCTGGTGG-3') and is located approximately once/ 5 min throughout the *E. coli* chromosome. Therefore, the chi sites have to be suppressed in order to use ExoV to purify AT-DNA from the chromosomal DNA. Fortunately, 10 mM Mg^{2+} can convert RecBCD to a non-specific dsDNA exonuclease (Yu *et al.*, 1998). So, before carrying out the experiment, it was very important to find an optimum working condition for ExoV in order that the enzyme could perform most efficiently to fully degrade linear ds-DNA.

The working concentrations of Mg^{2+} and ATP were varied to optimize degradation, while leaving circular DNA intact. 2μg of MG1655 chromosomal DNA was cut with EcoRV to obtain fragments of a suitable size to mimic fragmented chromosomal DNA. Plasmid pHPK-bp was added into the reaction system as a control, since ExoV should not cut circular DNA. The final concentration of Mg^{2+} was then set to 20mM for all subsequent work and 0.2 mM was chosen as the final concentration for ATP. To obtain the best digestion result, additional ATP was added at the indicated times during incubation (Table 3.4; Fig 3.7).

From reaction 1 to reaction 2, reactions were begun with 0.2mM of ATP, while in reaction 5, 0.5mM of ATP was added and 0.1mM in reaction 6. It is obvious that 0.1mM of ATP is not enough for ExoV to act efficiently, since the digestion of MG1655 DNA was very incomplete (Fig 3.5 R6). However, too much ATP is not a good choice either. My result (Fig 3.5 R5) confirmed previous observations that high concentration of ATP favours unwinding DNA rather than degrading it, since although MG1655 chromosomal DNA has been degraded completely in R5, the supercoiled plasmid DNA was reduced relative to other reactions, while the open circular plasmid DNA was increased. The digestions performed in reaction 2 and reaction 4 (Fig. 3.5 R2 and R4) were incomplete, suggesting that supplying additional ATP to replace consumed ATP is important, since it is inadvisable to provide too much ATP initially.

In this experiment, it seems that supply of additional ExoV did not make any difference. This is probably because ExoV is a quite efficient enzyme and the chromosomal DNA present was not too high. In addition, it is also very important to know that ExoV has little effect on circular DNA since large amount of supercoiled plasmid DNA remained intact, while the chromosomal DNA had been digested completely, though some supercoiled plasmid DNA was lost (R1, R3). This is possibly because supercoiled DNA is nicked by contaminating nucleases. The above experiment suggests that ExoV suits the purpose of the new protocol.

Table 3.4 Test of the nuclease activity of ExoV in different conditions.

	R6	R2	R4	R1	R3	R5
ATP	**0.1mM**	**0.2mM**	**0.2mM**	**0.2mM**	**0.2mM**	**0.5mM**
Time when additional ATP added	**at 60 min, 140 min**	**at 60 min**	**at 60 min**	**at 60 min, 140 min**	**at 60 min, 140 min**	**at 60 min, 140 min**
ExoV	**1μg**	**1μg**	**1μg**	**1μg**	**1μg**	**1μg**
Additional ExoV(1μg) added at 120min	**Yes**	**Yes**	**No**	**Yes**	**No**	**Yes**
Digestion result	**P.D.**	**I.D.**	**I.D.**	**C.D.**	**C.D.**	**C.D.** #

Note: MG1655 chromosomal DNA (2μg) which had been digested by EcoRV was used as targets. Plasmid pHPK-pb was added as circular DNA control. 10 x reaction buffers were added to all reactions along with 20 mM Mg^{2+} and 1μg ExoV. Additional ATP and ExoV were added at indicated time for different reactions. "**C.D.**" stands for Complete Digestion of the linearized chromosomal DNA; "**I.D.**" stands for Incomplete Digestion of the linearized chromosomal DNA and "**P.D.**" stands for Poor Digestion of the linearized chromosomal DNA. "#" means that although the linearized chromosomal DNA has been completely digested in R5, the supercoiled plasmid DNA is reduced, while the open circular plasmid DNA is increased. The results are explained in the text.

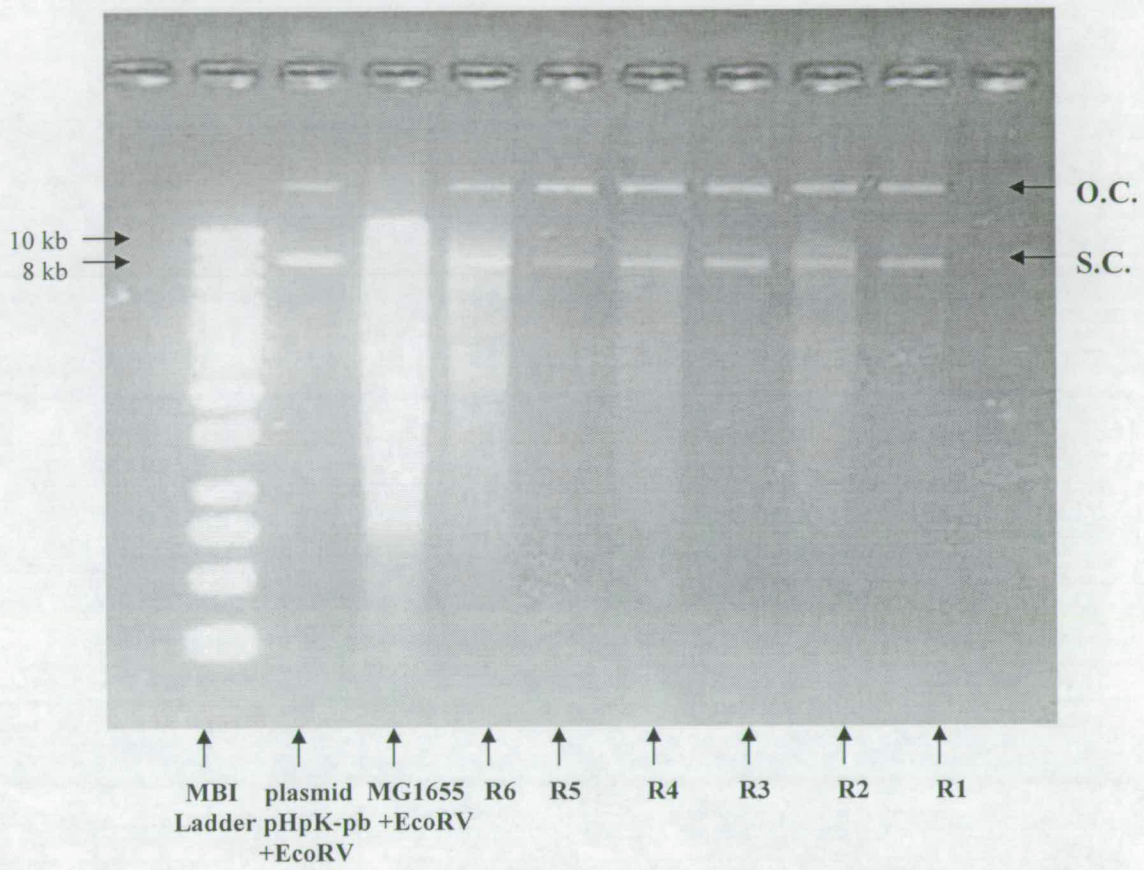


Fig 3.7 Test of nuclease activity of ExoV by agarose gel electrophoresis. R1-R6 are reactions listed in Table 3.1. EcoRV digested MG1655 DNA is a minus control. Plasmid pHPK-pb was used here as circular DNA control, therefore it should not be cut by ExoV. The conditions used for each reaction was list above in Table 3.1 and results were explained in the text.

3.4.2. Execution of the new protocol and discussion

Cells were infected by phage P1 as usual. In order to harvest as much abortively transduced DNA as possible, a relatively high multiplicity of infection (m.o.i.), up to 4, was used, but not greater than this, since cells will be lysed prematurely if m.o.i. is too high. After absorption of P1 into the cell, cells were spun down and resuspended in fresh L Broth without Ca^{2+} and grown in the presence of 0.1M PMSF as proteinase inhibitor till lysis occurred. The additional resuspension step is in order to remove Ca^{2+}, since ExoV will lose all nuclease activities in the presence of Ca^{2+}. Once cells were lysed, the content of cells would be released into the supernatant including AT-DNA, P1-sheared chromosomal DNA as well as some P1 DNA. Most of the P1 DNA will have been packaged into the P1 heads which will be separated from free DNA on a CsCl step gradient at a later stage. The cell debris including the chromosomal DNA was then removed by centrifugation at 30,000g at 4°C for 15min. It has been reported in Sandri's paper that over 90% of the host chromosomal DNA will be removed under this condition, while 40-60% of abortively transduced DNA will remain in the supernatant. After concentration of the original large volume to a working volume by Millipore Centricon plus-80, 80μg ExoV was added to the system and incubated at 37°C for 3 hr under the optimum condition selected above. Additional ATP was added at 30min, 60min and 140min. Thereafter, RNase (DNase-free) was added to remove RNA. The remaining DNA was removed from the interface of a CsCl step gradient, consisting of 2ml of 1.8g.ml Fluorinert FC-40 and 4ml of 1.5g.ml CsCl solution containing 0.5% (W/V) sarkosyl and 1mM EDTA. The recovered DNA should contain all circular DNA and any other DNA not degraded by ExoV. However, only AT- DNA was labelled by BU and would be detectable using anti-BrdU antibody. After dialysis against storage buffer, the recovered DNA was stored at 4°C until ready to be tested by antibodies.

The procedure is straightforward and easy to execute. But, some unpredictable problems occurred during the process. First of all, ExoV did not work as efficiently as it did in the preliminary experiment and undesired DNA was left in the system (see Fig 3.8). Many methods were tried in order to improve the nuclease efficiency of ExoV. Firstly, Ca^{2+} had been removed before ExoV was added. Secondly, before adding ExoV, dialysis of LB against ExoV storage buffer had also been tried in case the original supernatant contained inhibitors of ExoV nuclease ability. But I could not find any differences

n the nuclease activity of ExoV before and after dialysis, possible because the dialysis was not going efficiently, as the volume (about 20ml) of the supernatant is a bit large for dialysis. In case the digestion time was too short to degrade chromosomal DNA completely, aliquots were taken after digestion for 3 hours and incubation continued over night with additional ATP supplied. However, extension of the digestion time did not increase digestion significantly relative to 3 hours' digestion. Perhaps, unlike the preliminary experiment, the supernatant contains non-dialysable inhibitors. There is also much more DNA present in the supernatant than had been present in the preliminary experiment so that more enzyme and ATP might have been required.

There have been previous reports of using ExoV to enrich for circular DNAs. In 1973, Mukai and his collaborators (Mukai *et al.*, 1973) succeeded in isolating circular DNA from whole cellular DNA using ExoV. Furthermore, the QIAGEN large-construct kit which is marketed by QIAGEN Company to isolate genomic DNA-free BAC, PAC and P1 also use ExoV to achieve the goal. In Mukai's work, cells were harvested by centrifugation and resuspended in Tris.HCl before being lysed by lysozyme and proteins removed by pronase and phenol extraction. Also, before adding ExoV, the lysate was sheared using a syringe with a micropipette tip. All these steps ensure the system is suitable for ExoV to work as an efficient nuclease.

But the above steps are not suitable for use here. First of all, abortively transduced DNA is associated with a protein, so any methods that remove proteins in order to purify DNA cannot be used. Secondly, P1 DNA is so large, about 100kb, which harsh methods used to shear chromosomal DNA may also cause shearing of abortively transduced DNA. Thirdly, abortively transduced DNA is released into a large volume of supernatant, so it is impossible to change buffers and reduce the volume simply by spinning the cells down. ExoI was required for extensive degradation of linear DNA and added after ExoV digestion in Mukai's work, although the reason why addition of ExoI is required is not very clear. Unfortunately, the outcome of ExoV digestion was not improved much even with addition of ExoI in my experiment, suggesting that some factors in the system might affect the activities of the enzymes. In the procedure of the QIAGEN KIT, both protein-removing steps and centrifugation-resuspension steps are also involved (QIAGEN Large-Construct Kit Handbook). Even so, the kit did not work very well to remove the chromosomal DNA contamination as I tried to purify P1 plasmid DNA using this kit.

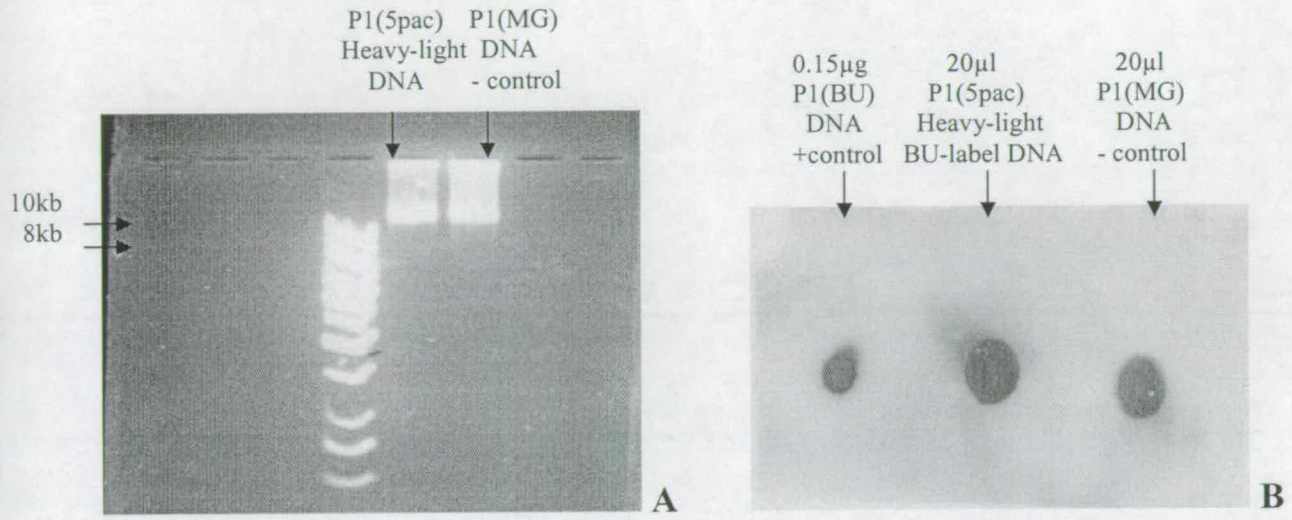


Fig 3.8 Extraction of AT-DNA using ExoV procedure. BU-labelled P1 (heavy→light) lysate made from MG1655 (5pac) was used to generate BU-labelled AT-DNA, while P1 (MG1655) lysate (no BU) was used as a negative control. After going through the procedure, the purified DNA was analyzed by electrophoresis and dot-blot. **A)** Electrophoresis analysis of DNA extracted using ExoV procedure. **B)** Dot-blot analysis of the extracted DNA using anti-BrdU antibody. As we can see, from the above result, too much DNA is in both final extracts and Anti-BrdU did not work well for this purpose since the minus control P1 (MG1655) DNA, which is not incorporated with BU shows a strong signal.

In addition, reconstruction experiments in which BU-labelled plasmid pUC19 were used to mimic AT-DNA was also carried out using the above ExoV procedure in order to see its efficiency. The procedure is the same as the one described above, except that instead of using BU-labelled P1(heavy-light) lysates to lyse cells, BU-labelled pUC19 was mixed with P1 (no BU) infected cells. However, these experiments suffered the same problem as described above - large amount of large sized DNA was left and could not be removed by ExoV (see Fig 3.9)

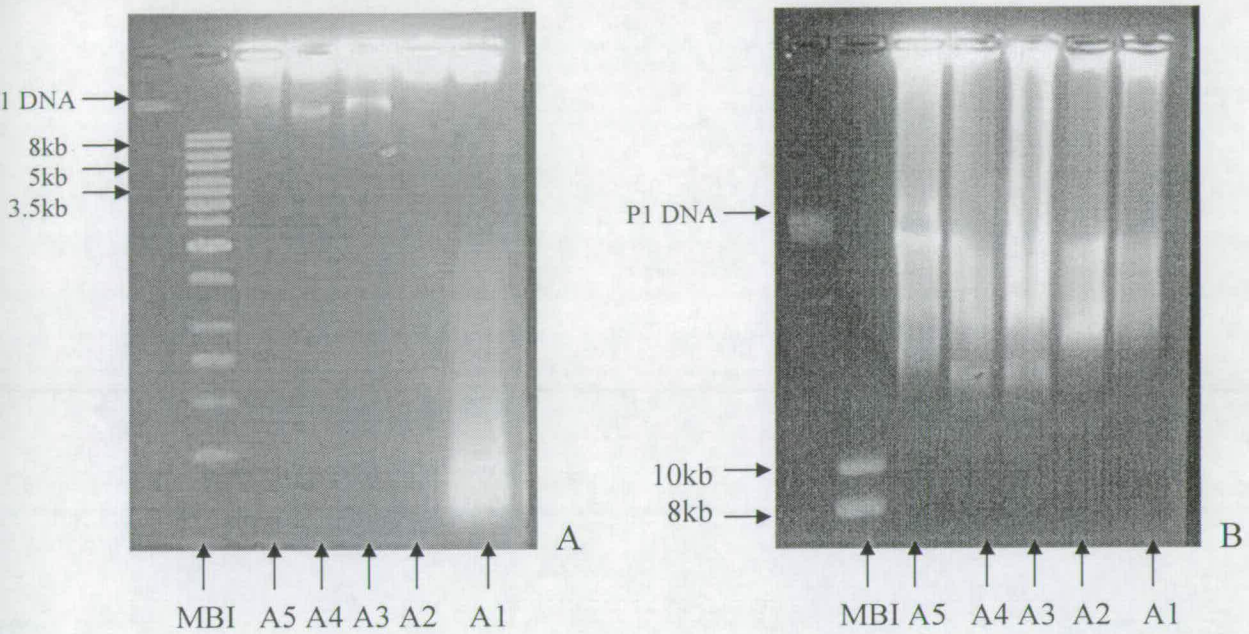


Fig 3.9 Electrophoresis analysis of DNA samples taken from different steps of the ExoV procedure. A1 (aliquot 1) is DNA taken after step 5 (see Table 3.3 above), A2 is DNA taken after step 8, A3 is DNA taken after step 9, A4 is DNA taken after step 10 and A5 is taken after step 11. **A)** The photo taken after running at 12 Volts for 24hrs. **B)** The photo taken after running at 60 Volts for 4 hours more.

From the above results, we can see that cellular DNA was not removed efficiently by the procedure. As mentioned above, one possibility is that a large amount of DNA was released from lysed cells so that the amount of ExoV added was not adequate. Besides chromosomal DNA, some phage DNA which was not packaged into a phage head may also avoid degradation by ExoV, since P1 DNA will be circularized soon after injection into the cell (see Introduction). This could be a serious source of contaminating DNA. In further work, a 5 *pac*-containing *recA* strain could possibly be used as the recipient to reduce DNA contamination. This is based on two possibilities 1) that the host chromosome might be easier to break down in a 5 *pac*-containing strain after P1 infection relative to that in MG1655, since there are 5 *pac* sites on the chromosome and therefore, become a good substrate for ExoV; 2) that most injected P1 DNA could not be circularized in *recA* cells and therefore, could be degraded by ExoV.

Secondly, detection of purified AT-DNA, if it were present, was also a big challenge. Since I have not labelled transducing DNA with ^{32}P, I could not purify the abortively transduced DNA from the bulk of DNA by CsCl equilibrium gradient centrifugation and analyze it by autoradiography. In the step gradient that I used above, all DNA that survived ExoV treatment will be retained. To distinguish the abortively transduced DNA from other DNA, blotting with anti-BrdU antibody is the only method available. The membrane-transferring step involved in the procedure will always result in losses reducing the signal intensity relative to autoradiography. Theoretically, only the abortively transduced DNA should be detected by antibodies since no other recovered DNA was labelled. This was supported by the test of the specificity of anti-BrdU antibody to BU-labelled DNA (Fig. 3.6). In the test, DNA from P1(5pac BU-labelled heavy-light) lysate gave much stronger signals than did the same amount of DNA from P1(MG1655 –control) lysate, suggesting that the specificity of the antibody is quite high (also see Fig 3.2, 3.3), even though we cannot rule out that the specificity of the antibody I used in this work is not high enough.

However, in the actual experiment using the AT-DNA enrichment procedure the result was disappointing. The hybridization signals for all samples are very strong and of similar intensity (Fig 3.8). It is possibly that the DNA extracted from the interface of the step gradient is a mixture, containing other DNA in addition to AT-DNA. Since, in Fig 3.6, unlabelled DNA binds some antibody, large quantities of extra unlabelled DNA cause serious background on the membrane, which will obscure the signal.

Overall, the protocol is theoretically feasible, but considerable further work will be needed to improve it. The major areas for improvement are:

1. ExoV will need to work better.
2. Chromosomal DNA will need to be removed more effectively. Some new approaches such as the one described above could be tried for this purpose.

This work was undertaken on the assumption that the work of Sandri and Berger is correct: i.e. that AT-DNA is a circle, the ends of which are held together by AT-protein. That this putative configuration of AT-DNA is correct is essential for the procedure attempted to be feasible since the entire protocol is built on this assumption. The hypothesis is derived solely from Sandri and Berger's

work (Sandri and Berger, 1980b), in which they observed a change in the sedimentation velocity of abortively transduced DNA in neutral sucrose gradients and in velocity of migration on agarose gels from a faster migrating form to a slower one after treatment with SDS or heating at 70°C. I am disappointed that I failed to either confirm or falsify their work.

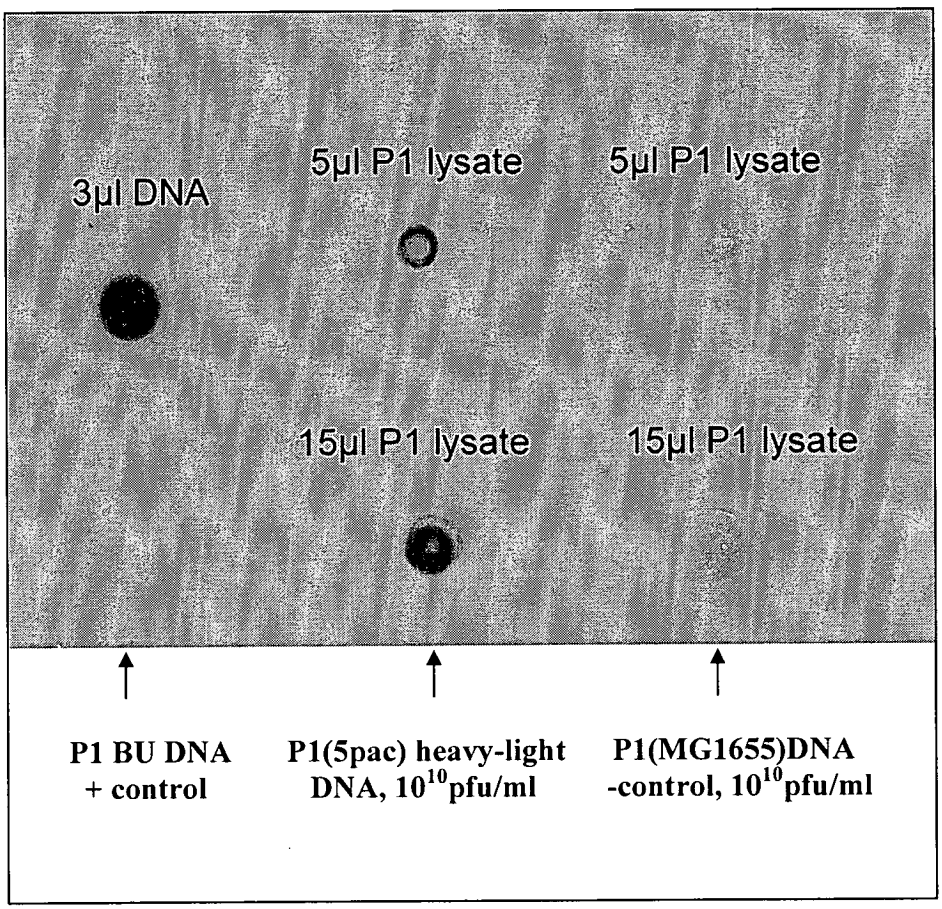


Fig 3.10 Western dot blotting to test the labelling efficiency of transducing DNA in P1(5pac, heav-light) lysate and the specificity of the anti-BrdU antibody.

3.5 A quick look at the *darA* operon of Bacteriophage P1

As described in Chapter one, some evidence has indirectly suggested a relationship between the P1 *darA* operon and AT-protein (Iida *et al.*, 1998; Yamamoto, 1982). Iida with his colleagues demonstrated that deletion of the *darA* operon from P1Cm2 will cause two distinct phenotypes in the phage, Gta^- (negative for generalized transduction affected, which means transduction of chromosomal markers with higher frequency than obtained with wild-type P1) and Tsu^- (negative for transduction stimulated by ultraviolet irradiation). Yamamoto has also shown that a P1 amber mutant, *sus50*, which maps within or near the *darA* operon, has the same phenotypes as the *darA* operon deletion mutant. The defects were complemented by plasmid pSHI93-Δ4, but not by plasmid pSHI95 (Iida *et al.*, 1998). The difference between these two plasmids is that plasmid pSHI93-Δ4 is carrying a fragment that extends from the *dar* promoter to the beginning of the VI open reading frame, while pSHI95 carries a fragment containing only the *lydA* and *lydB* genes from the operon (Fig 3.7 and Table 3.2). It is possible that the protein produced from the segment of the *darA* operon carried by pSHI93-Δ4 interferes with stable transduction so that UV irradiation of P1 particles can increase the transduction frequency only in the presence of this protein. This would be consistent with the protein being the mysterious abortively transduced protein which renders the transduced DNA abortive. If the above hypothesis were right, then over expression of this protein in the cell should reduce the transduction frequency of markers.

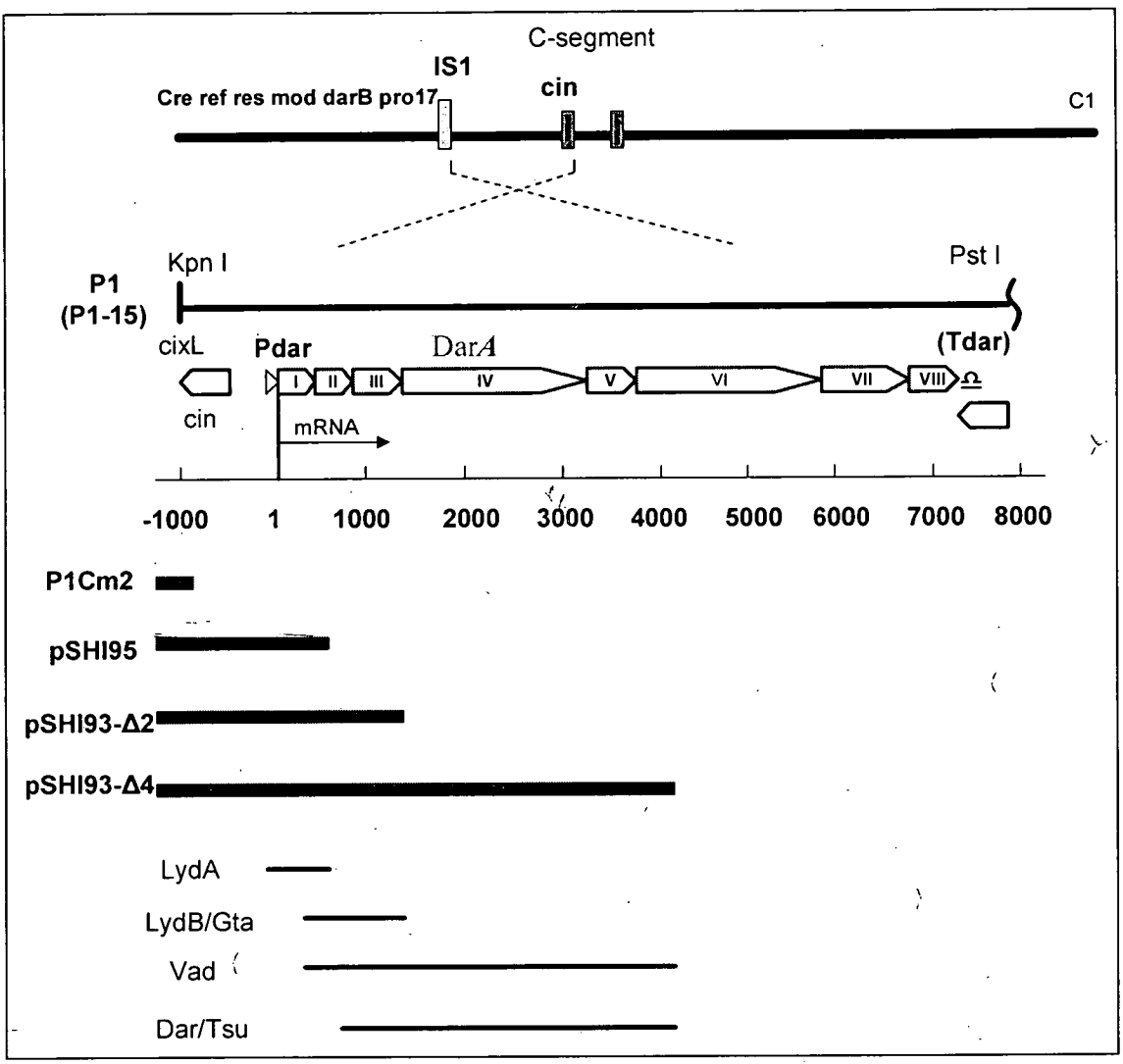


Fig 3.7 Location of the *darA* operon on the P1 genome (from Iida *et al.* 1998). ORFs deduced from the DNA sequence are represented by the open pentagonal arrows, and the P*dar* promoter for the *darA* operon is indicated by the filled triangle. The thick horizontal bars represent the DNA segments carried on the plasmids or by P1 mutant indicated. The linear thin horizontal bars represent the DNA segments carrying the accessory genes responsible for the indicated functions. Orf I = *lydA*, orf II = *lydB* and orf IV = *darA*.

Table 3.2 Summary of the phenotypes of the mutant phage P1Cm2 and complementation studies (from Iida *et al.*, 1998).

	Phenotypes			
Phage/plasmid	Gta	Dar	Tsu	Vad
P1Cm2	–	–	–	–
P1Cm2/pSHI95	–	–	–	–
P1Cm2/pSHI93-Δ2	+ -	–	–	+ -
P1Cm2/pSHI93-Δ4	+	+	+	+

Note: the wild type and mutant phenotypes are indicated by + and -, respectively, and + - represents a leaky mutant phenotype. P1Cm2 phages grown in the presence of plasmid for complementation studies are indicated by P1Cm2/plasmid. Gta stands for generalized transduction affected; Tsu for transduction stimulated by ultraviolet irradiation; Vad for viral architecture determinant and Dar for defense against restriction.

In order to test this hypothesis I tried the following experiments. Plasmid pSHI93-Δ4 was transformed into both MG1655 and MM303 cells. P1 lysates were made from the plasmid-containing MG1655 and named as P1(pSHI93-Δ4). As a control, P1(pBR322) was made from pBR322-bearing MG1655, since plasmid pSHI93-Δ4 is a pBR322-based plasmid. In the first experiment (Table 3.6, Fig 3.8), both donor and recipient cells harboured the plasmid. In agreement with expectation, the presence of the *darA* operon segment appeared to reduce transduction about 10-fold. To determine whether extra DNA in donor or recipient is responsible for this, I repeated the experiment testing all four possible combinations of plasmid/no plasmid in donor and recipient.

Table 3.6 Transduction frequencies of *ilv* and *metB* by P1(pSHI93-Δ4) and P1(pBR322)

			P1(pSHI93-Δ4)	P1(pBR322)
Titer			2×10^{10} pfu/ml	4×10^{10} pfu/ml
Recipient			MM303(pSHI93-Δ4)	MM303
MARKERS	*ilv*	**Colonies on plates**	60	1402
		No. of colonies./ml	6000	140200
		No. of trans./phage	3×10^{-7}	3.5×10^{-6}
	metB	**Colonies on plates**	13	206
		No. of colonies./ml	1300	20600
		No. of trans./phage	6.5×10^{-8}	5.2×10^{-7}

Note: MM303(pSHI93-Δ4), the pSHI93-Δ4 containing MM303, was infected by P1(pSHI93-Δ4), while MM303 was infected by P1(pBR322).

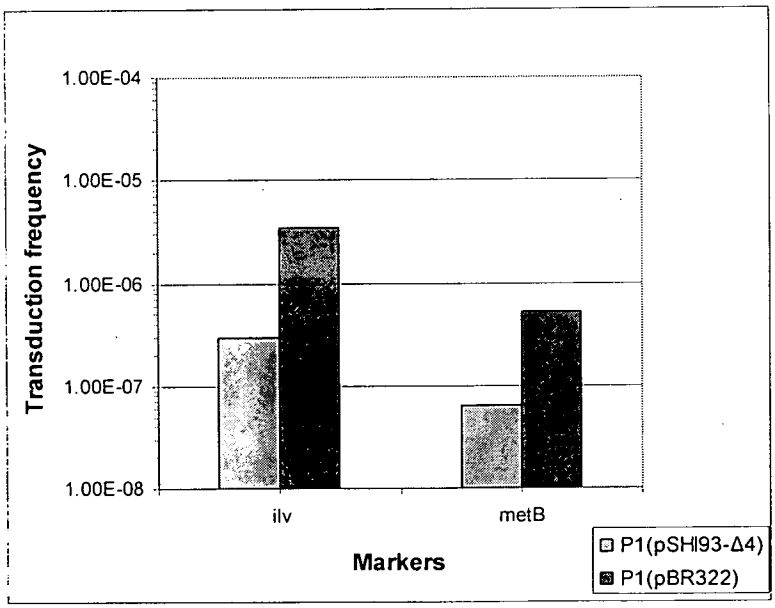


Fig 3.8 Transduction frequencies of *ilv* and *metB* by P1(pSHI93-Δ4) and P1(pBR322). (see table 3.6)

Table 3.7 Transduction frequencies of *trp* and *metB* by P1(pSHI93-Δ4) and P1(pBR322)

		P1(pBR322) – **donor -**				P1(pSHI93-Δ4) – **donor +**		
Titer		4×10^9 pfu/ml				2×10^9 pfu/ml		
Recipient		MM303 **(pSHI93-Δ4)**		MM303 **(control)**		MM303		MM303 **(pSHI93-Δ4)**
Experiment No.		**1**	**2**	**1**	**2**	**1**	**2**	**2**
trp	**No.(plate)**	269	129	332	146	30	15	39
	No. / ml	2690	1290	3320	1460	300	150	390
	No. /phage	**6.7×10^{-7}**	**3.2×10^{-7}**	**8.3×10^{-7}**	**3.7×10^{-7}**	**1.5×10^{-7}**	**7.5×10^{-8}**	**1.9×10^{-7}**
metB	**No.(plate)**	79	109	121	226	18	22	26
	No. / ml	790	1090	1210	2260	180	220	260
	No. /phage	**2.0×10^{-7}**	**2.7×10^{-7}**	**3.0×10^{-7}**	**5.7×10^{-7}**	**9.0×10^{-8}**	**1.1×10^{-7}**	**1.3×10^{-7}**

Note: both MM303 and MM303(pSHI93-Δ4), the pSHI93-Δ4 containing MM303, were infected by P1(pSHI93-Δ4) and P1(pBR322).

From Table 3.7, we can see that: 1) the presence of extra *dar* fragment in the donor does not affect phage titer significantly; 2) the presence of extra Dar fragment in the recipient when it was absent from the donor may reduce transduction frequency slightly (not significantly in this experiment); 3) the presence of extra Dar fragment in the donor appears to reduce transduction several fold. It is not possible from this experiment to say whether fewer transducing phages in a P1 lysate are made in the presence of extra Dar fragment or they fail to recombine (i.e. more abortives transduced).

3.5.1 Discussion

We showed in section 3.5 that having the *dacA* operon containing plasmid pSHI93-Δ4 in both donor and recipient cell reduces transduction. In order to find out whether this effect result from *dacA* expression in the donor cell or in the recipient cell transductions were carried out using donor either with or without the plasmid to prepare lysates. These lysates were then used to transduce MM303 with or without plasmid. As shown in Table 3.7, it seems that the interaction could have happened before infection. In addition, the effect on transduced DNA made by expressing the protein from plasmid indicates that the protein is probably a DNA-binding protein which acts in *trans*, though we cannot completely rule out the possibility that the protein interacts with DNA indirectly by interaction with one or more intermediates.

Which of the genes in the *dar* operon is responsible for decreased transduction? The segment of the *darA* operon on plasmid pSHI93-Δ4 contains the first 5 complete open reading frames in the *darA* operon, including the *dar* promoter, plus the beginning part of gene VI. Considering the complementation result of pSHI93-Δ2 and pSHI95 (Fig 3.7 and Table 3.2), only the sequence ranging from *lydB* to gene VI gives rise to the Tsu phenotype. In the sequence, only gene III, gene IV (*darA*) and gene V are complete open reading frames, while gene II (*lydB*) and gene VI are only partial. Therefore, the candidate for this protein is narrowed to three genes: gene III, *darA* and gene V. However, one possibility that we cannot rule out is that instead of one gene function, the Tsu phenotype is contributed by interaction between products of genes over the segment carried by pSHI93-Δ4. In this case, the product of any genes on the fragment is needed to be considered in addition to that of one or more than one of the above three candidates for the contribution of Tsu phenotype.

Gene I (*lydA*) and Gene II (*lydB*) are involved in controlling lysis (Iida, *et al.*, 1998 and see introduction for detail). Gene III and gene V are function-unknown genes (Lobocka, M., personal communication). Each gene encodes a rather small protein. The product of gene III is a 203aa protein, while gene V encodes a 121aa protein. The product of the *darA* gene is a 68kD DNA-binding protein located within the phage head. It defends any DNA packaged into a P1 head against restriction by type I restriction endonucleases. It is injected into the host cells along with the DNA and enables DarB,

I restriction endonucleases. It is injected into the host cells along with the DNA and enables DarB, another P1 antirestriction protein, about 200kD in size, to function (Iida, *et al.*, 1987). Considering that DarA protein is a DNA-binding protein, it seems the most likely of these to be AT-protein. However, several lines of evidence make this possibility unlikely. Firstly, DarA was found in purified heads isolated from a P1*sus*50 lysate, suggesting that DarA is not the protein Yamamoto mutated. Secondly, DarA was not likely to be the protein that Ikeda and Tomizawa (Ikeda and Tomizawa, 1965b) found covalently attached to the end of transducing DNA in transducing particles, because DarA is found in particles containing phage DNA, and because it seems to be easily removed from DNA by phenol treatment (Iida *et al.*, 1987). The Ikeda and Tomizawa result does not rule out the possibility that DarA is AT-protein, although DarA appears in both transducing and infective particles. In addition, although Ikeda and Tomizawa showed that the protein is covalently attached to the end of DNA, Sandri and Berger (Sandri and Berger, 1980b) showed that abortively transduced protein can be removed by treatment with SDS (0.25%) and incubation for 20min at 70°C.

If DarA is not the protein missing in the *sus50* mutant which decreases abortive transduction, the only two genes left on pSHI93-Δ4 which are included on pSHI93-Δ2 are gene III and gene V. The problem for them is each of them produces a very small protein, which is inconsistent with the protein found in Ikeda and Tomizawa's work (Ikeda and Tomizawa, 1965b), in which the protein was calculated to be as big as 500kD. It is possible that abortive transduced protein is a complex rather than a single protein. Thus, as mentioned above, many products of gene III or gene V could associate with themselves or with other proteins to form this complex. Therefore, it is worth investigating these two genes further seeing whether there are interactions between one or both of these gene products with other products of other genes on the segment. For instance, we could construct plasmids containing various combinations of these genes, and transform into donor and recipient cells, and then measure the transduction frequency.

Overall, the above result is consistent with the previous hypothesis that the protein from the *darA* operon has a role in breaking the balance between stable transduction and abortive transduction. It is not excluded that the Dar protein is the abortive transduced protein found associated with the ends of abortive DNA, rendering them resistant to degradation by the host nucleases.

Chapter IV

Studies on packaging initiated at *pac* sites on the chromosome

4.1. Introduction

In this project, experiments were carried out to study the mechanism of packaging of chromosomal DNA by bacteriophage P1.

Originally, a *pac* insertion was made by replacing the *argB* gene with a *pac* site in order to produce more P1 transducing particles. Transduction and titration showed that the inserted *pac* site successfully increased the transduction frequency of markers close to it. More information about the effect of the *pac* site on packaging of chromosomal DNA was obtained by Southern blottings. When I decided to abandon my attempt to purify abortively transduced protein, I chose instead to examine the effects of multiple chromosomal *pac* site insertions on transduction and P1 production.

To achieve this goal, up to 5 *pac* sites alone or in combination have been introduced into the chromosome using the pKO3 gene replacement procedure (Link A.J., *et al.*, 1997; Merlin C., *et al.*, 2002). Although a single *pac* site had been put into the chromosome by Sternberg *et al.* and, separately, by Hanks as part of another replicon (N. Sternberg and J. Coulby 1987a; Hanks, PhD thesis, 1986), this method allowed us to place the *pac* site alone into the chromosome at prechosen positions. This minimizes the possibility that effects due to insertion of vector DNA or insertion in a deleterious position will be mistakenly considered as an effect of *pac* insertion.

Firstly, the effects of a single *pac* site insertion were analyzed with both physiological and molecular biological approaches. Transduction frequencies for chromosomal markers close to *pac* increased by as much as several 100-fold, while Southern blotting of encapsidated chromosomal DNA shows that the effect of the inserted *pac* site in increasing chromosomal DNA packaging extends for at least 5 P1 genome lengths.

Later, multi-*pac* insertions were studied using similar approaches. Two striking phenomena were observed in strains bearing 2 or more *pac* sites. Firstly, the time when cells start to lyse after P1 infection is significantly delayed in multi-*pac*-containing strains while WT and one-*pac*-containing strain lyse at the normal time. Secondly, strains which have 2 or more *pac* sites give very poor lysates; plate lysates prepared on these strains are 10^4-fold lower in titer than all WT (or the one-*pac*-

containing strain) lysates and single cycle liquid lysates are also reduced in titer. These observations suggest that there is competition between the packaging from *pac* sites on the chromosome and on the P1 genome. Southern blotting of the DNA packaged from this strain reveals that each of the *pac*-sites stimulates packaging of markers nearby.

4.2. One *pac* site on the chromosome

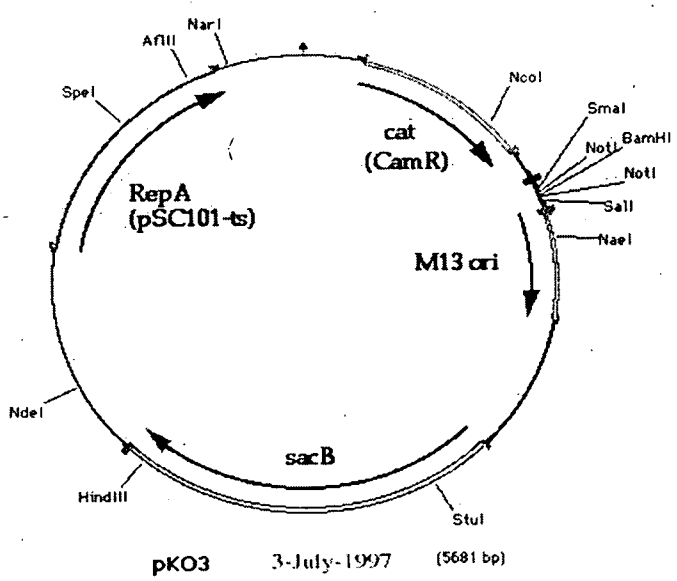
4.2.1. *pac* replacing the *argB* gene

4.2.1.1. Construction of the pKA2 vector and replacement of *argB*

In contrast to P22, phage P1 transduction frequencies vary among different chromosomal markers by only about 30-fold (Masters, 1977) and nearly all of the differences can be explained by gene dosage differences in the donor cells or the stability of the transduced DNA in recipient cells (Newman and Masters 1980; Masters *et al.*, 1984). Further work done by Hanks *et al.* showed, using quantitative Southern hybridisation (Hanks *et al.*, 1988), that most chromosomal markers are packaged at a similar level. He also demonstrated that there are no sequences homologous to the *pac* site on the chromosome, suggesting that the packaging of chromosomal markers by P1 probably initiates from many sites all over the chromosome instead of from a few located at specific places. However, when a *pac* site on a λ vector was put into the bacterial chromosome at the *attB* site by site-specific recombination, an increase in the transduction frequency of chromosomal markers to one side of the fragment by as much as 80-fold compared to a non-lysogenic control, was observed (N. Sternberg and J. Coulby 1987a). Hanks (Hanks, PhD thesis, 1986) observed a 3-fold increase in the transduction frequency of markers near the *pac* site, when he directed a *pac* site on pBR325 to the chromosome of a *polA* strain by homologous recombination. Because of the difference in magnitude of transduction frequency changes seen in these two approaches, we could not know if the λ sequence flanking the *pac* site plays a role in the stimulation of transduction frequency of the chromosomal markers seen in the lysogen. It is also possible that more than one *pac* site was inserted into the bacterial chromosome during lysogenization. On the other hand, the method used by Hanks probably resulted in unstable plasmid-chromosome recombinants and thus plasmids were easily excised from the chromosome. In addition, *polA* deficiency could itself affect some aspect of the packaging process. Our initial strategy was therefore to use recent technical advances to target a *pac* site to specific locations on the bacterial chromosome. Our initial choice was to replace the *argB* gene, since it would then be easy to screen *pac*-inserted strains by screening for arginine auxotrophy and also many markers nearby could later be used to test the effects of the inserted *pac* site on transduction frequency.

The pKO3 plasmid (Fig 4.1) was used to carry out this replacement, because of two important features. First of all, pKO3 is not maintained at high temperature, because of its temperature sensitive RepA protein. This important feature allows us to select for recombination easily at high temperature. At the nonpermissive temperature, plasmid maintenance requires integration into the chromosome by homologous recombination and creates a tandem duplication. This provides the opportunity for excision with allele exchange after return to the permissive temperature. Secondly, to select for loss of the plasmid sequence from the cell, the *B. subtilis* gene *sacB* is incorporated into the plasmid, since the product of *sacB* in the presence of sucrose is lethal to *E. coli* (Link *et al.*, 1997).

The 650 bp P1 EcoRI – 20 fragment was cut from the pPAC plasmid with EcoRI. The Klenow fragment (from DNA polymerase I) was used to fill in the ends and the fragment was then cloned into the pre-blunted SacI site between the fragments flanking the *argB* gene (Fig. 4.3) in pHM2. pHM2 had previously been generated by using crossover-PCR to join *argC* and *H* and inserting the fragment into the pKO3 plasmid at the BamHI site (Fig. 4.2). Pfu polymerase was used in this work to ensure the maximum fidelity of copying.



The features of pKO3:

1. Temperature sensitive pSC101 RepA^{ts} plasmid cannot replicate at 42°C.

2. *SacB* gene product is lethal to cells in the presence of sucrose.

3. Chloramphenicol resistant.

Fig. 4.1 The pKO3 vector and its features

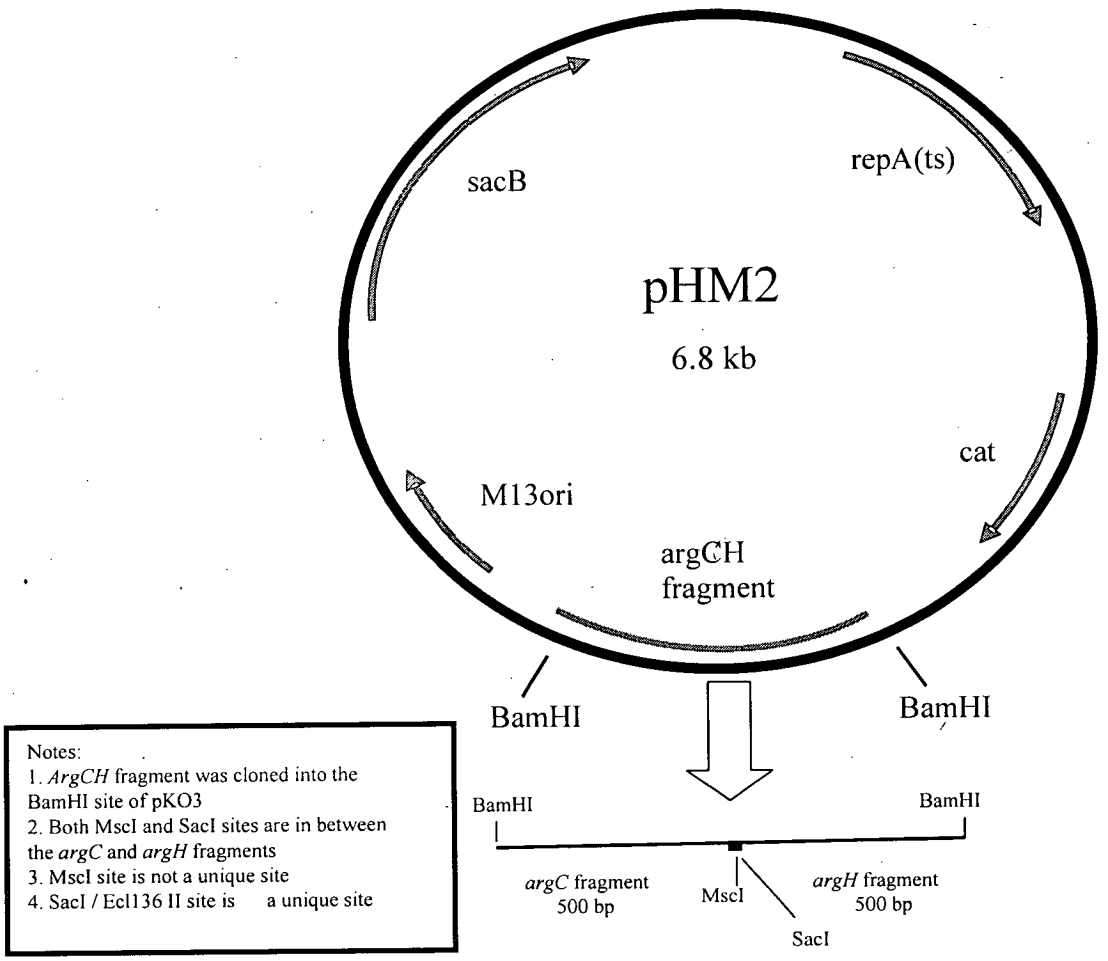


Fig. 4.2 pHM2, the *argB* -deletion vector

The *pac*-containing plasmid was then transformed into the recipient cell, at the non-permissive temperature, 42°C, to force the vector to integrate into the chromosome, thus creating an unstable tandem duplication. After shifting from non-permissive temperature to the permissive temperature, 30°C, the integrated plasmid can be excised from the chromosome in one of two ways, either to exactly reverse the original insertion-event or to exchange alleles with the chromosome creating a pKO3 – chromosomal DNA hybrid plasmid and a *pac*-containing *argB*-deleted chromosome (Fig. 4.4). Using sucrose plates to take advantage of the toxicity of *sacB*, we could select the plasmid-cured strain, which either still contains *pac* or is wild type. In the construct we seek, the *argB* gene will be knocked out and replaced by the *pac* fragment thus generating an auxotrophic strain, which can be screened on arginine free plates (Fig. 4.5). In addition to the *pac*-inserted strain, an *argB*-deleted strain was also made as a control using the same procedure, but with pHM2 as the replacement plasmid.

Theoretically, after resolution and loss of vectors from the cell, the ratios of recovered wild-type strains and that of recombined strains should be 1:1. However, in fact, 10 *argB* deleted colonies were obtained from 52 survivors, while only 1 *pac*-inserted colony was obtained from 106. The reason why there was this difference is not very clear. One possibility is that the cell is not happy with *pac* on the chromosome. However, the growth curve test performed later (see Fig. 4.1D) showed that the *pac*-containing strain has a growth rate similar to that of the wild type strain, suggesting that, at least after integration into the chromosome, *pac* has very little effect on cell growth. We cannot rule out the possibility that before *pac* was successfully integrated into the chromosome, cells are disadvantaged by the plasmid. Another possibility is that the size of the insert affects the efficiency of integration, since I have observed that using the same procedure, it is much easier to insert a PCR *pac* site (about 130bp) into the chromosome relative to insertion of P1 EcoRI-20 fragment (about 650bp). In addition, the position of the replaced gene may affect the efficiency of integration as well, since Dr. Merlin in our lab had found that different sized inserts had a similar efficiency of integration.

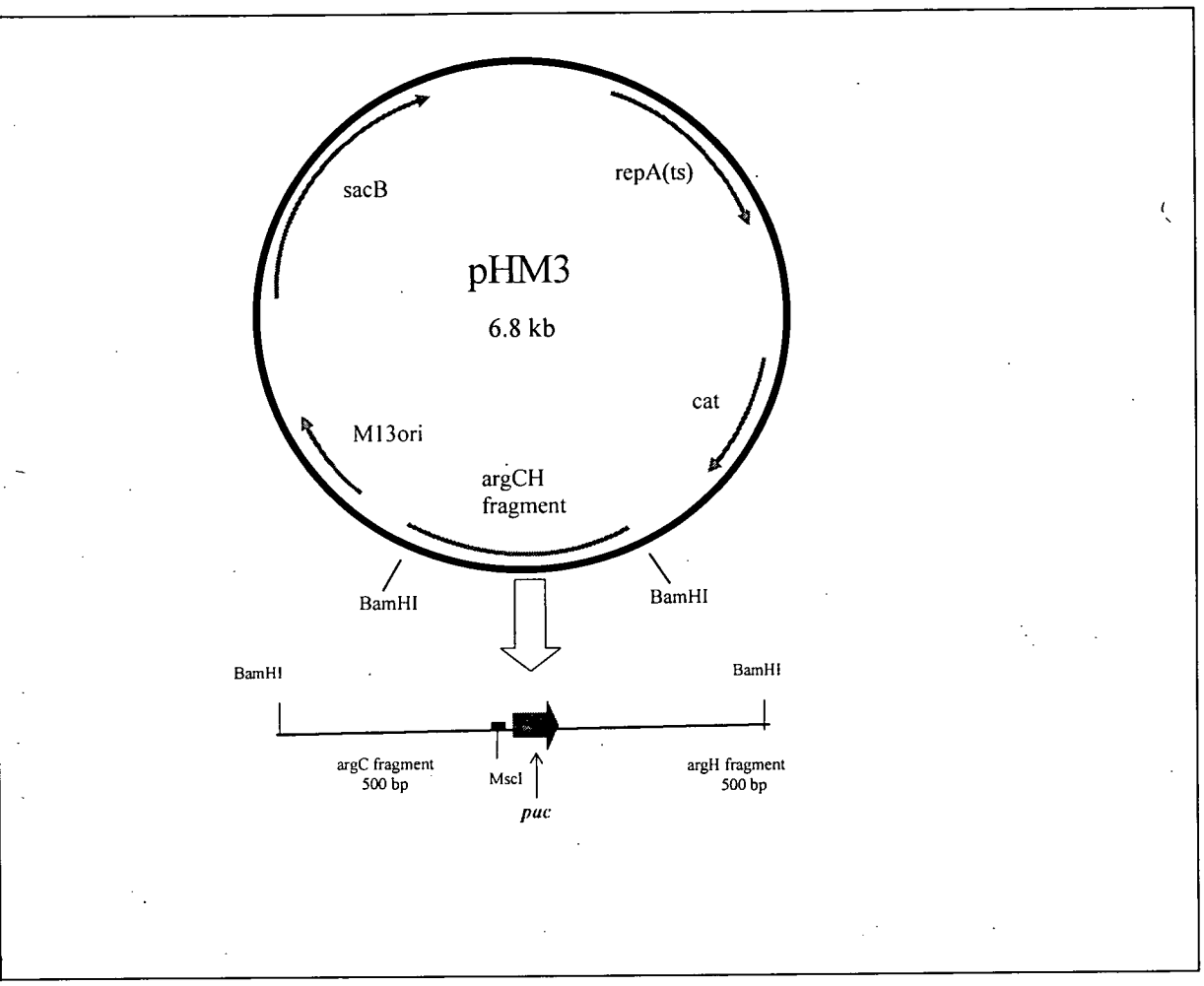


Fig 4.3 pHM3, the *argB* -replacement vector

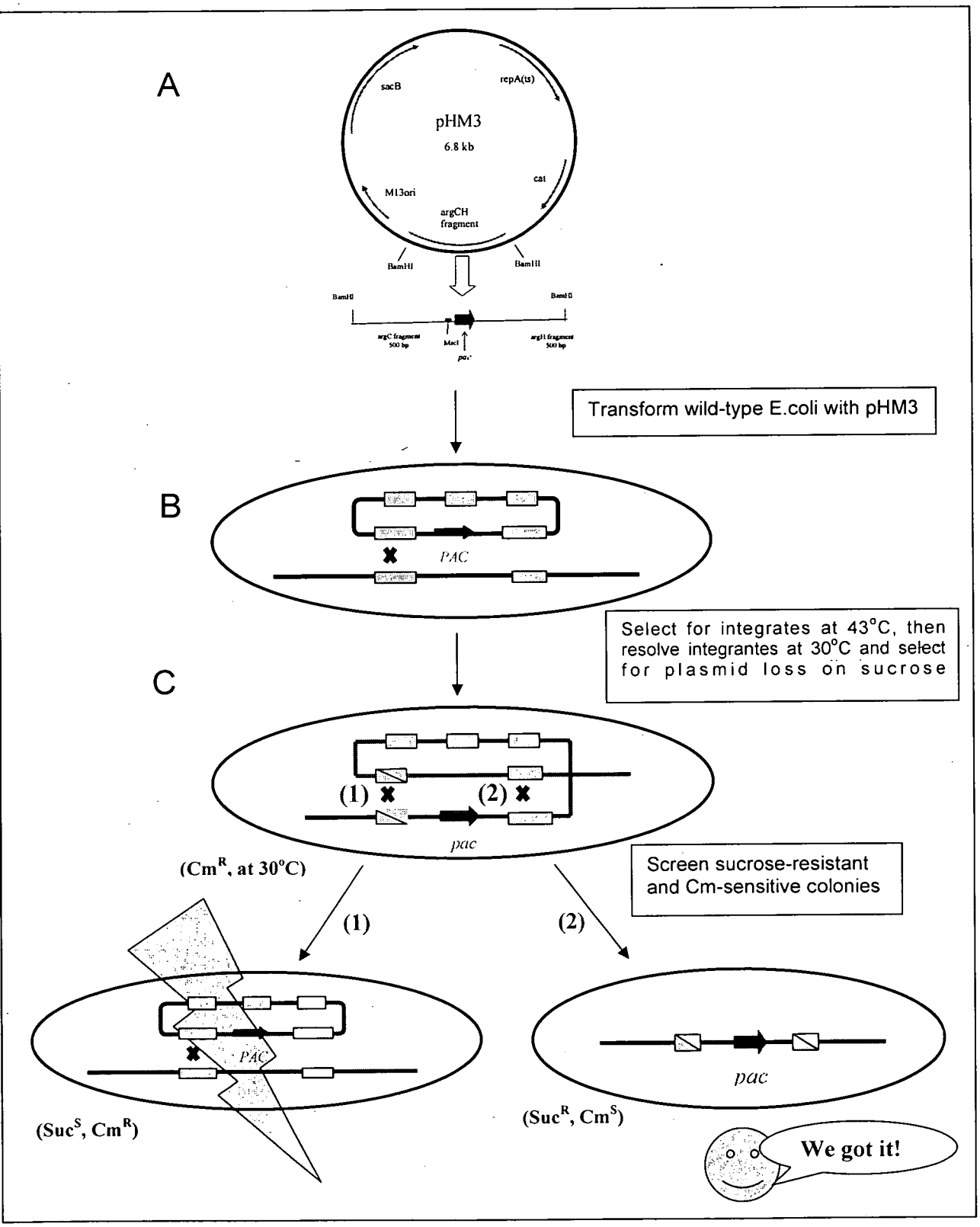


Fig 4.4 Cartoon of the protocol for replacement of *argB* with pKO3

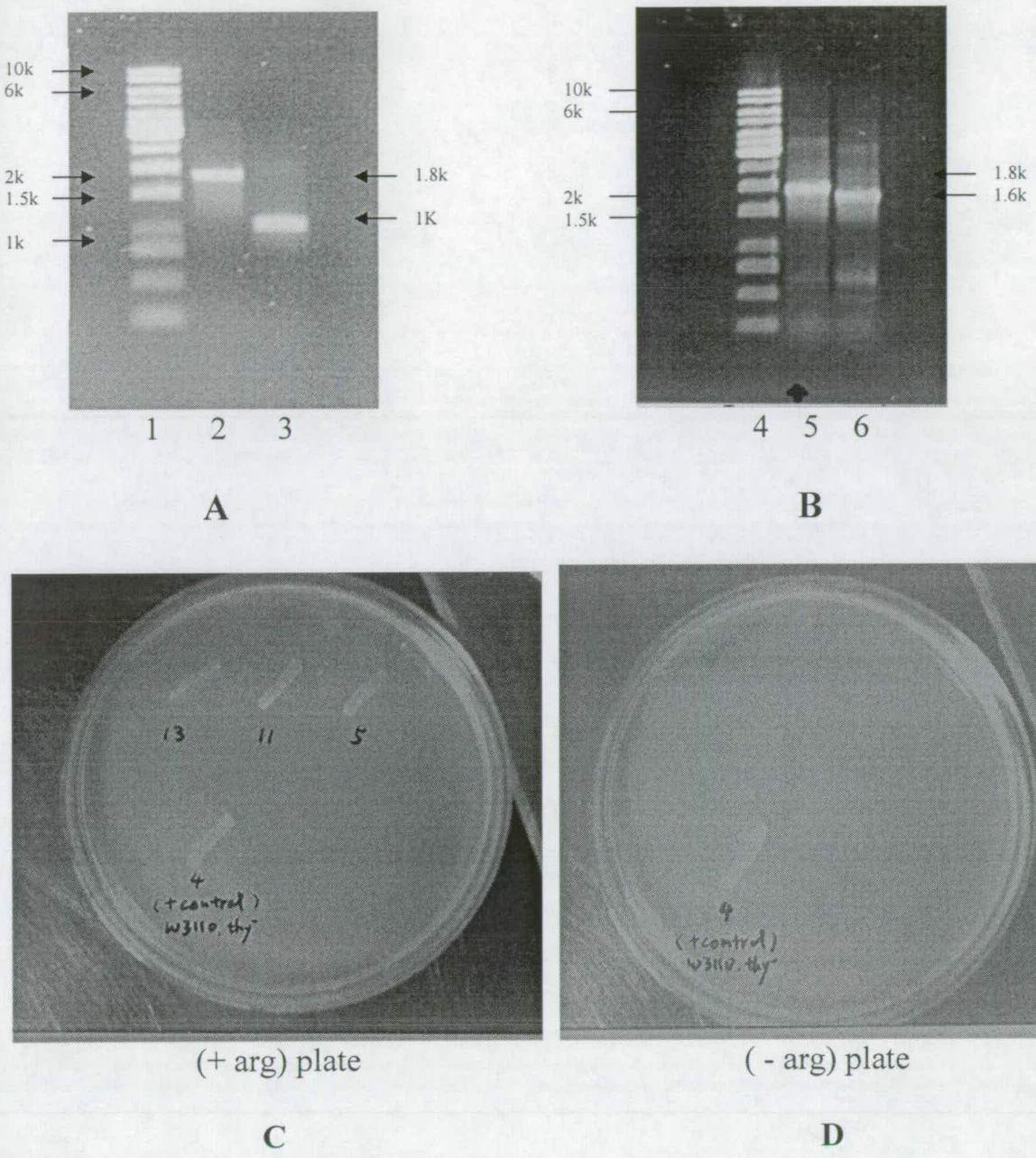


Fig. 4.5 *argB* deleted and replaced by *pac* fragment. A) Gel electrophoresis of PCR products made using outer-primers for crossover PCR to amplify the *argB* region of the parent strain (lane 2) and the *argB* deleted strain (lane 3). MBI 1kb markers were separated in Lane 1. B) *argB* is replaced by the P1 *EcoR*I -20 *pac*-containing fragment. MBI 1kb marker, PCR of wild-type strain and PCR of the single *pac*-containing strain (see p 133) are shown in lanes 4, 5 and 6, respectively. C-D) Test of the *pac*-containing arginine auxotrophic strains after screening. The plates were VB glucose with arginine (C) or without arginine (D). Colony 5, 11 and 13, all restreaked from single colonies, were *pac*-containing arginine auxotrophic strains. Colony 4 is a positive control.

4.2.1.2. The presence of *pac* increases the transduction frequency of chromosomal markers to one side of pac

Transduction was carried out to see if the transduction frequency of the chromosomal markers was increased by the integrated *pac* fragment lacking any sequences other than P1 sequences. The optimum ratio of phage to cells had been determined by plotting the number of transductants against the P1 titers. A titer of or below 5-6 x 10^8 pfu/ml was obtained by dilution of P1 stock lysates with phage buffer or LB in most transduction experiments to ensure that the killing of cells by P1 would be minimized. Lysates were made from both MG1655 and *argB* <> *pac* strains, and termed P1 (MG) and P1 (*argB* <>*pac*) respectively. The *metB* gene was chosen to be a transduction marker, since it is about 0.1min away from the *argB* locus and therefore should be affected significantly by *pac*. *trpA*, was another marker tested, since its location is far from the *pac* site. The transduction results are shown below (Table 4.1 and Fig 4.6). As expected, the *pac* site on the chromosome increased the transduction frequency of *metB* by several hundred fold relative to the *pac*-free strain, while the transduction frequency of *trpA* was similar in both strains, confirming the results of previous work done separately by Sternberg and Hanks. It is interesting to note that *pac* can increase transduction about 10-fold more than Sternberg observed. We cannot rule out the possibility that the extra sequence, such as λ sequence, which are neither from P1 or host DNA may stimulate transduction from *pac*. However, it is more likely that the location of the *pac* site on the chromosome is important. Also, it is obvious that the distance between *pac* and the marker tested will greatly affect the transduction result.

Table 4.1 Transduction frequencies of *metB* and *trpA* in both *pac*-free and *pac*-containing strains

Recipients	MM303							
Selection markers	***metB***				***trpA***			
Strains of P1	**P1(MG)**		**P1(arg<>pac)**		**P1(MG)**		**P1(arg<>pac)**	
Titer of P1(pfu/ml)	$5x10^8$ (L1)	$6x10^9$ (L2)	$3x10^8$ (L1)	$6x10^9$ (L2)	$5x10^8$ (L1)	$6x10^9$ (L2)	$3x10^8$ (L1)	$6x10^9$ (L2)
No. of colonies (from plates)	**169**	**379**	**584**	**657**	**128**	**338**	**77**	**197**
No. of colonies (multiply by dilution)	**169**	**379**	**58400**	**657000**	**128**	**338**	**77**	**197**
Multiplied No./ml	**1690**	**3790**	**584000**	**6570000**	**1280**	**3380**	**770**	**1970**
No. /phage (Tran. Freq.)	**$3x10^{-6}$**	**$6x10^{-7}$**	**$2x10^{-3}$**	**$1x10^{-3}$**	**$2.6x10^{-6}$**	**$5x10^{-7}$**	**$3x10^{-6}$**	**$3x10^{-7}$**
Average No./phage	**$1.8 x10^{-6}$**		**$1.5 x10^{-3}$**		**$1.6 x 10^{-6}$**		**$1.65 x 10^{-6}$**	
Met/*trp*	**1.1**		**909**		--------		---------	
Ratio to P1(MG)	----------		**833**		--------		**0.92**	

Note: two lysates which were made at different times (with different titers) were used for each P1 lysate. P1(*argB* <>*pac*) was diluted 100-fold or 1000-fold depending on the titer, in transduction of *metB*. Two set of lysates, P1(MG) and P1(*argB* <>*pac*) made at the different time were used and labelled as (L1), lysates No. 1 and (L2) lysates No.2.

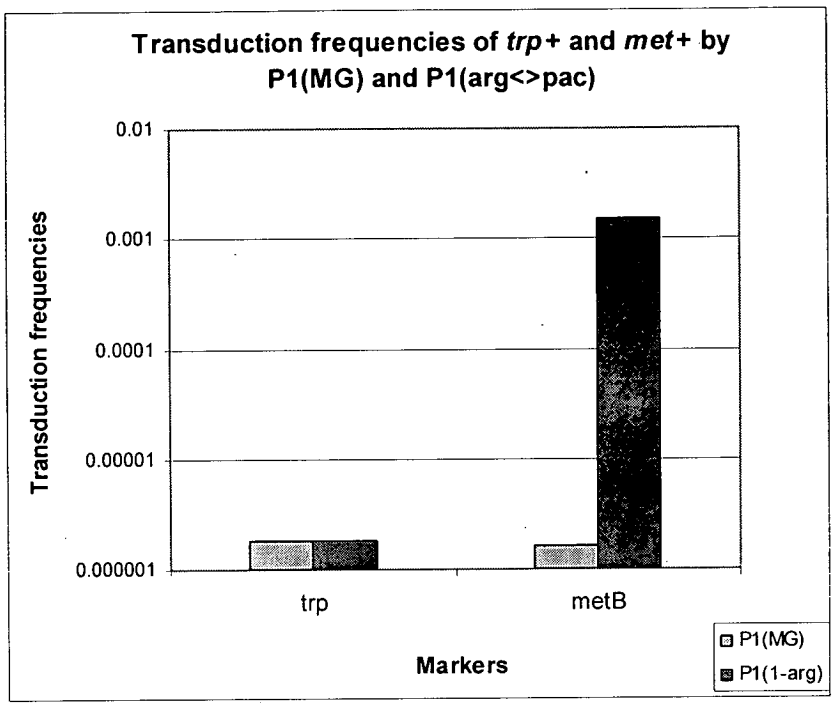


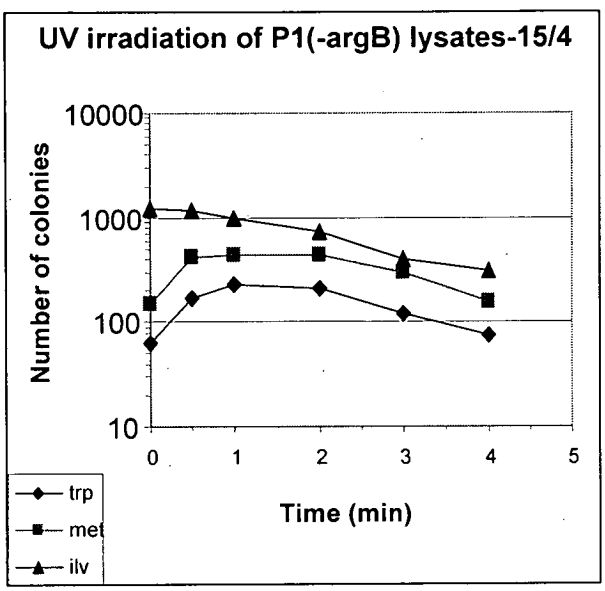
Fig 4.6 Transduction frequencies of *met*B and *trp*A in both *pac*-free and *pac*-containing strains

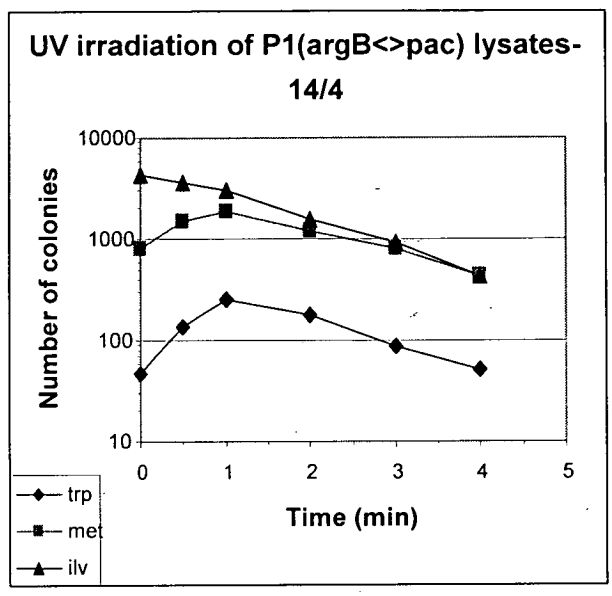
4.2.1.3. Biological evidence that the increased transduction frequencies observed in lysates prepared on MG1655 *argB*< >*pac* are not solely the result of the conversion of abortive to complete transductants: UV Irradiation of P1 Lysates

After transduction about 90% of transduced DNA will stay in cells in a form refractory to both degradation and recombination, yielding what are termed abortive transductants. Abortively transduced DNA does not replicate or recombine and so remains extrachromosomal and is transferred to only one daughter cell at cell division (Gross and Englesberg, 1959; Ozeki, 1959; Lederberg, 1956). More recent work suggests that abortively transduced P1 DNA may be maintained because proteins bind its ends together (Sandri and Berger, 1980b). It is thus possible that the increased numbers of e.g. Met^+ transductants we obtain may result not only from increased packaging of transducing DNA but also from a decrease in abortive transductant formation, perhaps as a result of insufficient binding protein being made for all the DNA being packaged. In order to distinguish between these possibilities we employed UV irradiation of P1 lysates. It has been known for a long time that abortive transductants can be converted to stable transductants by irradiation with small doses of ultraviolet (Benzinger and Hartman, 1962; Wall and Harriman, 1974). Previous work done by Newman in our lab has shown that UV irradiation of either transducing lysates or recipient cells results in a selective stimulation of the transduction of markers which are normally transduced poorly (Newman and Masters, 1980). Although the mechanism by which UV acts on the transduction process is still uncertain, it has been hypothesised that small doses of UV damage the target DNA. Once the damaged DNA is introduced into the recipient it becomes a substrate for recombination. Maximum post-irradiation transductant stimulation for Met^+ progeny observed by Newman and Masters was 4-fold. We would therefore expect a similar level of stimulation if the met^+ DNA transduced in these experiments forms a normal proportion of abortive forms.

To do this P1 lysates were made from the arg<>*pac* strain and *pac*-free *argB* strain, named P1(*pac*) and P1(-*arg*B) respectively. The lysates were irradiated with low doses of UV as described in Materials and Methods and aliquots were taken at the indicated times and used to transduce MM303. *metB* and *trpA* and *ilv* markers were transduced. Note that the transduction frequency of *ilv*, which is located close to the origin and always has a very high transduction frequency (Masters and Broda 1971), is not increased by UV irradiation, possibly because no or very few transducing DNA

fragments containing *ilv* fail to recombine in the recipient cell. As expected, the transduction frequency of *ilv* was not stimulated by UV, while the stimulation curves for *met*$^+$ and *trp*$^+$ transduction by P1 (*pac*) parallel those for *met*$^+$ and *trp*$^+$ transduction by P1 (-*arg*B), indicating a comparable ratio of abortive to complete transductants, suggesting that the total amount of abortively transduced DNA has been increased (Fig 4.7, Table 4.2). The maximum ratios of increase of transduction frequencies by UV observed by Newman (Newman, B.J. and Masters M., 1980) are 4-fold and 3-fold for *metB* and *trpA* marker respectively. This is slightly different from my observation where I observed that transduction frequencies of *metB* and *trpA* of a P1(*-argB*) lysate were increased by 2.9-fold and 3.6-fold respectively after UV irradiation, while a 5-fold increase for *trp*A and a 2.3-fold increase for *metB* were observed in a P1(*pac*) lysate.





A B

Fig 4.7 The effects of UV stimulation on *met*$^+$ and *trp*$^+$ transduction: **A**) UV irradiation on P1 (*pac*) lysates. **B**) UV irradiation on P1 (*-argB*) lysates. The upper curve, *ilv*$^+$ marker; the middle curve, *met*$^+$ marker; the lower curve, *trp*$^+$ marker.

Table 4.2 Stimulation by UV irradiation of *Trp* and met transduction by P1(*-argB*) and P1(*pac*) lysates

Time (Min) UV	**trpA** colonies on the plate		**metB** colonies on the plate		**Ilv** colonies on the plate	
	P1(-*argB*)	P1(*pac*)	P1(-*argB*)	P1(*pac*)	P1(-*argB*)	P1(*pac*)
0	64	48	152	785	1203	4283
0.5	173	137	412	1492	1158	3613
1	231	259	437	1822	995	3090
2	206	180	435	1197	743	1570
3	118	88	293	812	408	915
4	76	52	153	427	308	433
Ratio of max. increase	3.6	5.4	2.9	2.3		

Note: 10^9pfu/ml of each lysate was irradiated in this work.

4.2.1.4. Using Southern blots to directly measure the effects of the *pac* site in chromosome on the packaging of the chromosomal DNA

No previous work at the molecular level has shown that the increase in transduction resulting from *pac* insertion in the chromosome is due to increased packaging of chromosomal DNA downstream from *pac*, although our work and Sternberg's work have demonstrated an increase in the transduction frequency of markers proximal to *pac*. We therefore have used quantitative Southern hybridization here to show that the amount of chromosomal DNA packaged into P1 heads is actually increased as well as to confirm that the *pac* site has an orientation, that packaging is unidirectional, and that the packaging frequency of markers is dependent on their distance from *pac*.

P1 DNA was purified from P1 (*argB<>pac*) and P1 (MG1655) lysates, cut with EcoRV and fragments separated on an agarose gel suitable for transfer to a membrane and Southern blotting performed as described in Materials and Methods. Alternatively, the gel was denatured and neutralized before transfer (see Materials and Methods). 26 probes were made in total for genes spaced along the entire chromosome (Fig. 4.8; Table. 4.3). Probes for use in a particular experiment were labelled together using the Roche High prime kit as indicated in Materials and Methods. In the vicinity of *pac*-sites, 2 minute spacing of probes was used where possible; otherwise 5 min spacing was preferred. Digested chromosomal DNA was included on the gels to enable calculation of relative probe labelling, and the quality of hybridization.

The filter was exposed overnight in a cassette and scanned with a Molecular Dynamics Phosphoimager the next day. It was immediately obvious that the DNA encoding certain markers was more highly represented within P1 (*argB<>pac*) DNA than that within P1 (MG1655) DNA (Fig. 4.9, 4.11). Data were quantitatively analyzed using Imagequant software.

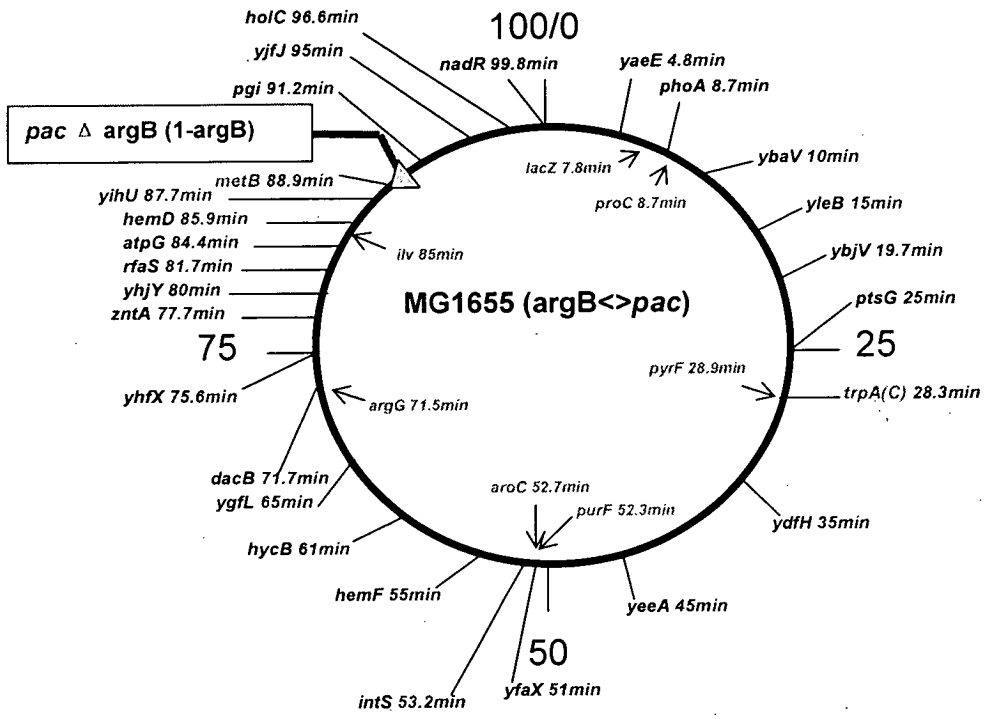


Fig 4.8 Chromosomal markers used in transducing studies. The triangle shows the position of the first *pac* site inserted into the chromosome and is pointing as packaging will proceed. The genes indicated outside the ring (followed by their position in min on the chromosome) were used for Southern probes. The markers inside the ring were used as transductional markers.

Table 4.3 Markers used for preparing Southern blot probes, in order of packaging

Marker names	location on chromosome	size by EcoRV
pac **Δ argB-(1-arg)**	**89min**	
metB	88.9min	8.0k
yihU	87.7min	2.4k
hemD	85.9min	2.0k
atpG	84.4min	13.5k
rfaS	81.7min	1.4k
yhjY	80min	1.7k
zntA	77.7min	10.8k
yhfX	75.6min	3.0k
dacB	71.7min	3.8k
ygfL	65min	2.5k
hycB	61min	3.1k
hemF	55min	13.5k
intS	53.2min	4.7k
yfaX	51min	1.4k
yeeA	45min	1.8k
ydfH	35min	2.2k
*trp*A(*trp*C)	28.3min	5.9k
ptsG	25min	6.7k
ybjV	19.7min	2.3k
yleB	15min	4.6k
ybaV	10min	1.8k
phoA	8.7min	9.7k
yaeE	4.8min	1.2k
nadR	**99.8min**	**3.0k**
holC	**96.6min**	**0.7k**
yjfJ	**95min**	**1.2k**
pgi	**91.2min**	**4.6k**

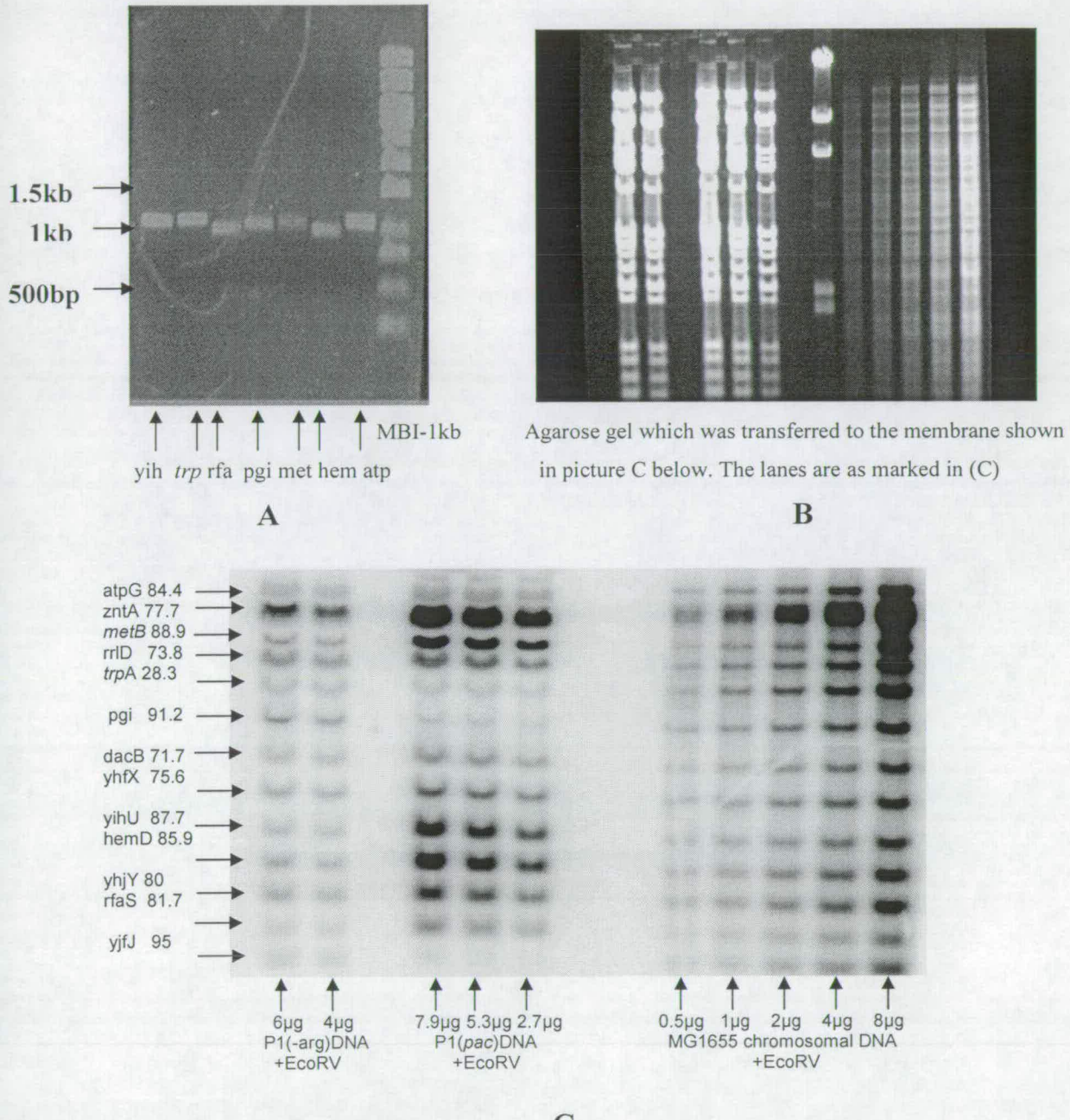
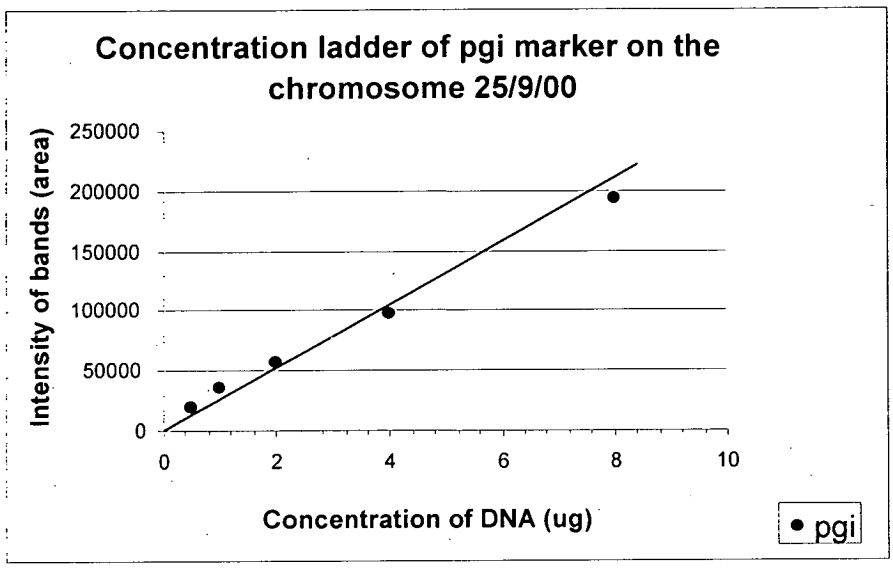
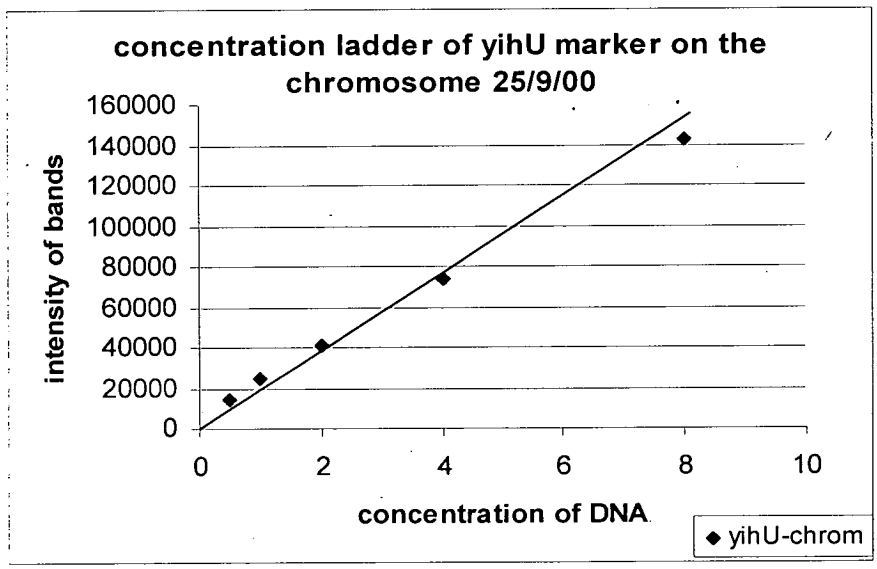


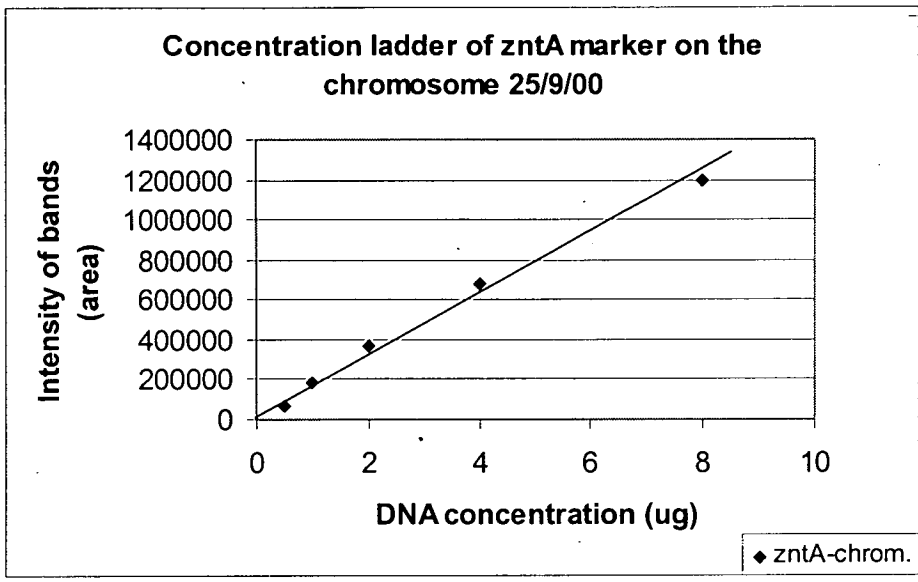
Fig 4.9 Southern blot to measure DNA packaged from MG1655 *argB<>pac* and parent **A**) Purified PCR products of a selection of the genes used to make probes. **B**) 0.8% agarose DNA gel of P1 and chromosomal DNAs cut with EcoRV. **C**) Final Southern blot. The filter was hybridized with probes and autoradiographed overnight. The markers visualized in the13 visible bands, are listed on the left-hand side. lane1: 6µg of P1 (*-argB*) DNA, lane2: 4µg of P1 (*-argB*) DNA; lane3: 7.9µg of P1(*argB<>pac*) DNA, lane4: 5.3µg of P1(*argB<>pac*) DNA, lane5: 2.7µg of P1(*argB<>pac*) DNA; lane6-10: MG1655 chromosomal DNA: 0.5µg, 1µg, 2µg, 4µg and 8µg respectively.


Concentration ladder of pgi marker on the chromosome 25/9/00
Intensity of bands (area)
250000
200000
150000
100000
50000
0
0
2
4
6
8
10
Concentration of DNA (ug)
pgi

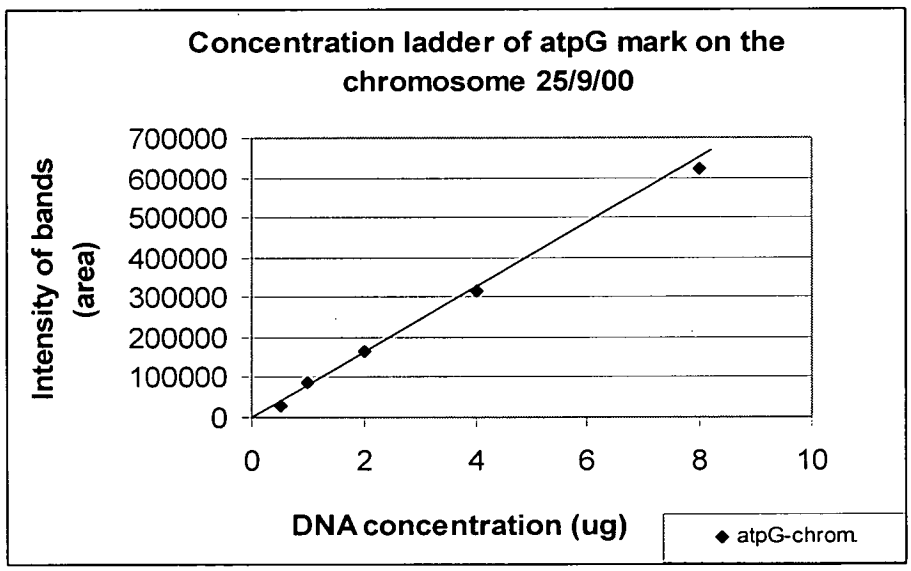
A


concentration ladder of yihU marker on the chromosome 25/9/00
intensity of bands
160000
140000
120000
100000
80000
60000
40000
20000
0
0
2
4
6
8
10
concentration of DNA
yihU-chrom

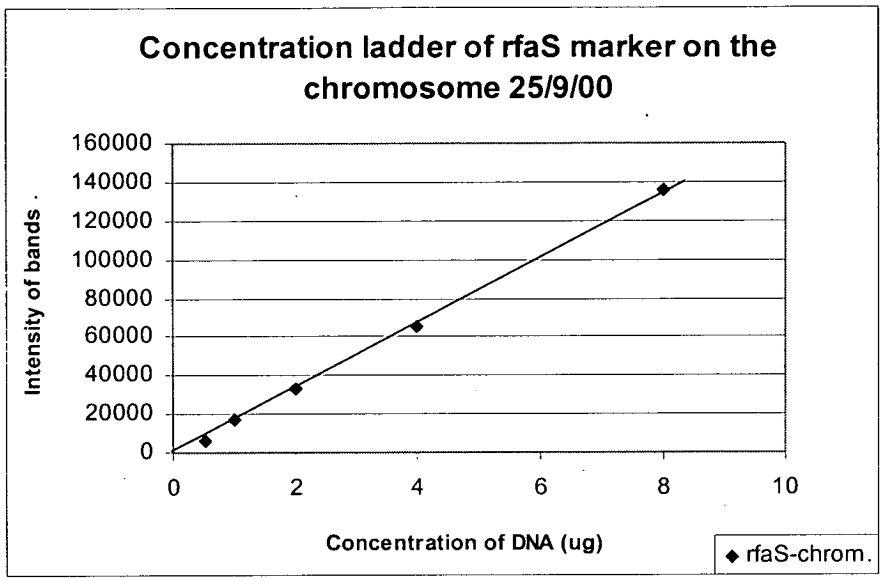
B


Concentration ladder of zntA marker on the chromosome 25/9/00
Intensity of bands (area)
1400000
1200000
1000000
800000
600000
400000
200000
0
0
2
4
6
8
10
DNA concentration (ug)
◆ zntA-chrom.

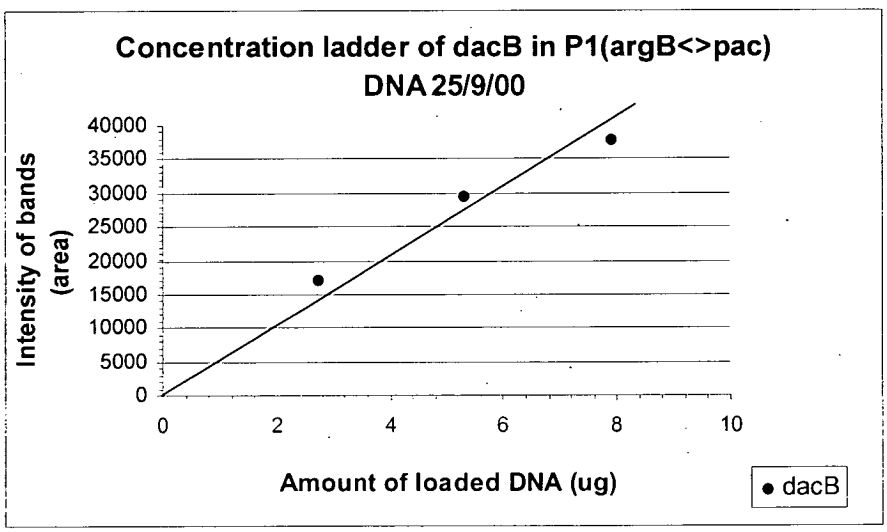
C


Concentration ladder of atpG mark on the chromosome 25/9/00
Intensity of bands (area)
700000
600000
500000
400000
300000
200000
100000
0
0
2
4
6
8
10
DNA concentration (ug)
◆ atpG-chrom.

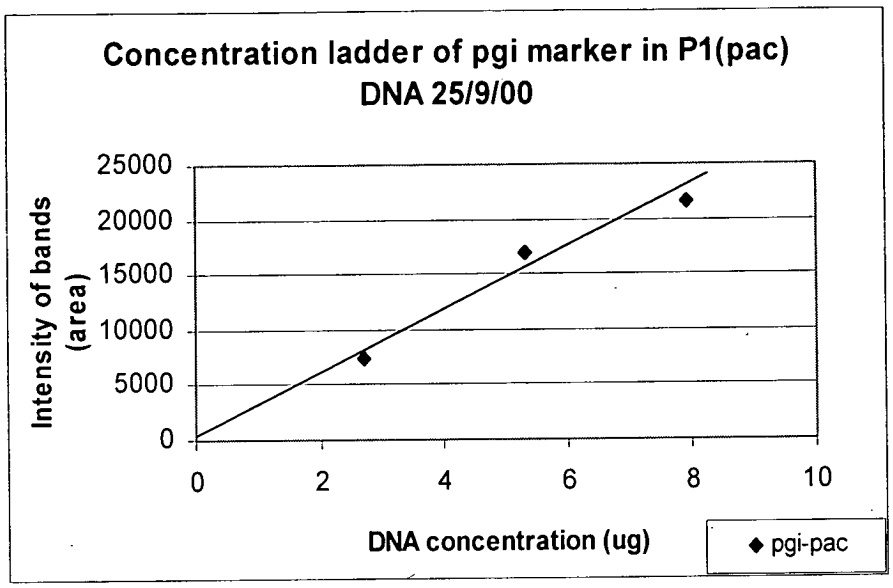
D


Concentration ladder of rfaS marker on the chromosome 25/9/00
Intensity of bands
160000
140000
120000
100000
80000
60000
40000
20000
0
0
2
4
6
8
10
Concentration of DNA (ug)
rfaS-chrom.

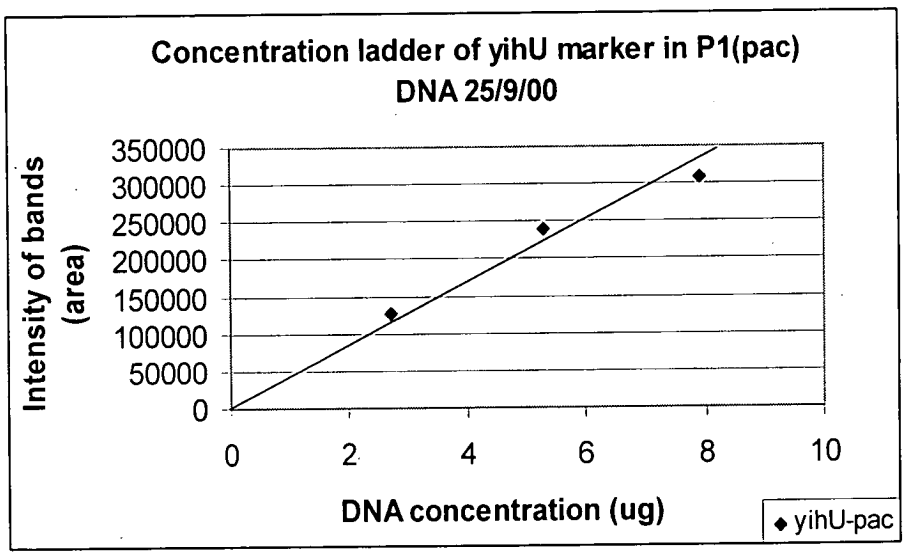
E


Concentration ladder of dacB in P1(argB<>pac) DNA 25/9/00
Intensity of bands (area)
40000
35000
30000
25000
20000
15000
10000
5000
0
0
2
4
6
8
10
Amount of loaded DNA (ug)
dacB

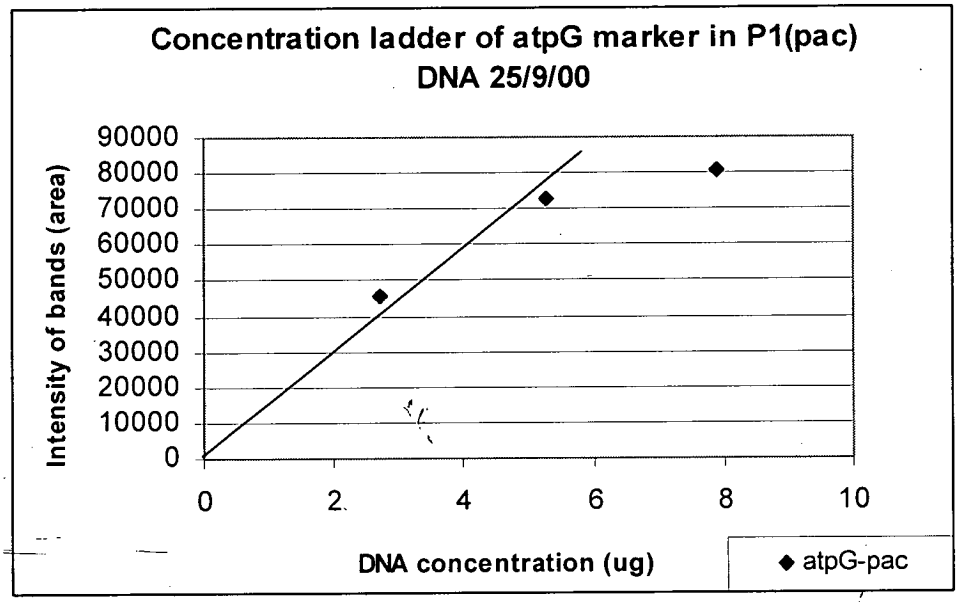
F


Concentration ladder of pgi marker in P1(pac)
DNA 25/9/00
Intensity of bands (area)
25000
20000
15000
10000
5000
0
0
2
4
6
8
10
DNA concentration (ug)
pgi-pac

G

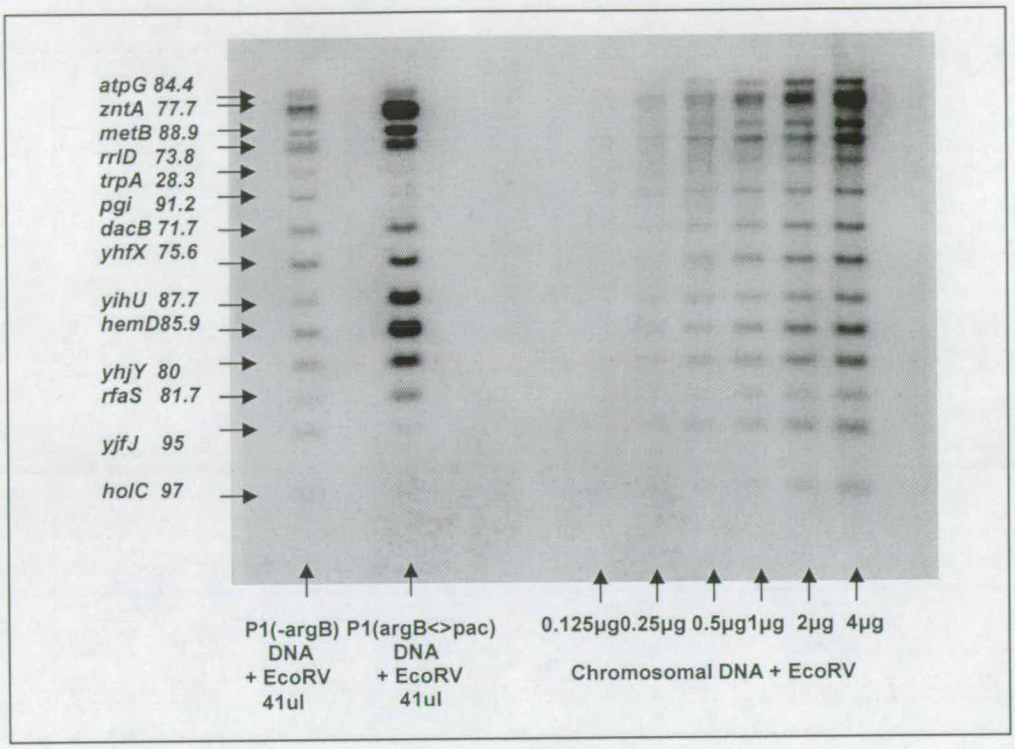

Concentration ladder of yihU marker in P1(pac)
DNA 25/9/00
Intensity of bands (area)
350000
300000
250000
200000
150000
100000
50000
0
0
2
4
6
8
10
DNA concentration (ug)
yihU-pac

H

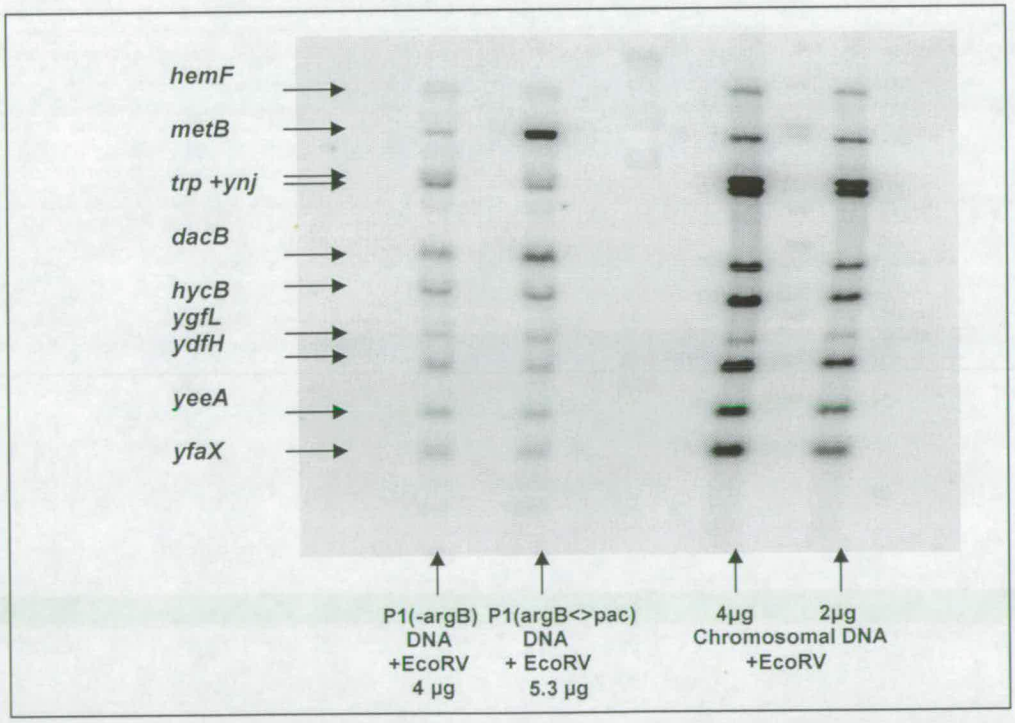


I

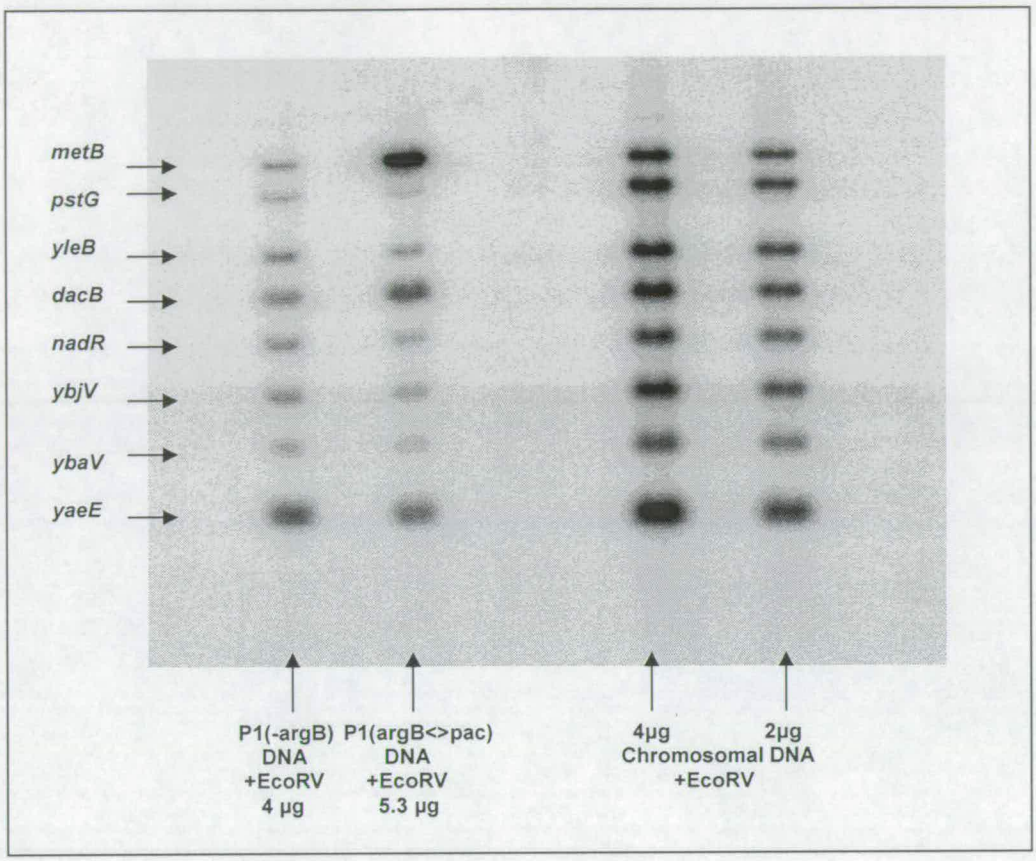
Fig. 4.10 Analysis of the DNA ladders of 25/9/00 Southern blot. The density of the bands was calculated with Imagequant. **A-D**) The chromosomal DNA concentration ladder (see Fig 4.9): 0.5μg, 1μg, 2μg, 4μg and 8μg respectively. The densities of bands were measured and plotted against the concentration of the chromosomal DNA. **E-H**) The P1 (*argB<>pac*) DNA ladder (see Fig 4.9): 2.7μg, 5.3μg, 7.9μg respectively. The densities of bands were measured and plotted against the concentration of of the P1(*argB<>pac*) DNA.


atpG 84.4
zntA 77.7
metB 88.9
rrlD 73.8
trpA 28.3
pgi 91.2
dacB 71.7
yhfX 75.6
yihU 87.7
hemD85.9
yhjY 80
rfaS 81.7
yjfJ 95
holC 97
P1(-argB) DNA + EcoRV 41ul
P1(argB<>pac) DNA + EcoRV 41ul
0.125µg
0.25µg
0.5µg
1µg
2µg
4µg
Chromosomal DNA + EcoRV

A


hemF
metB
trp +ynj
dacB
hycB
ygfL
ydfH
yeeA
yfaX
P1(-argB) DNA +EcoRV 4 µg
P1(argB<>pac) DNA + EcoRV 5.3 µg
4µg
2µg
Chromosomal DNA +EcoRV

B



C

Fig 4.11 Additional phosphorimager scans of Southern blots. Blotting protocol was the same as in Fig 4.9 (see Materials and Methods). **A,** another Southern blot (carried out on **29/08/00**), using the same set of probes presented in Fig 4.9. **B,** Southern blots using different set of probes (carried out on **1/03/01**). **C,** Southern blots using different set of probes (**30/04/01**). All the probes combined together cover the entire chromosome.

Table 4.4 Summary of the calculation of the ratio of P1(*argB*<>*pac*) DNA to P1(-*argB*) DNA

Dates	Markers	band size	position	Band intensity P1(arg<>*pac*)	Band intensity * P1(-argB)	(*pac*) / (-argB)	mean ratio
29/08/2000	*holC*	0.7k	97	5666	4329	**1.3**	**0.9**
P1lysates	*yjfJ*	1.2k	95	1472	4841	**0.3**	**0.5**
31/07/2000	*pgi*	4.6k	91	3061	12340	**0.2**	**0.4**
	metB	8.0k	89	48251	8899	**5.4**	**8.0**
	yihU	2.4k	88	52540	13026	**4.0**	**10.1**
	hemD	2.0k	86	84916	12761	**6.7**	**9.7**
	atpG	13.5k	84	13911	11025	**1.3**	**1.9**
	rfaS	1.4k	82	11049	5846	**1.9**	**4.5**
	yhjY	1.7k	80	31182	11627	**2.7**	**4.8**
	zntA	10.8k	78	94011	34821	**2.7**	**3.2**
	yhfX	3.0k	76	24553	20111	**1.2**	**2.9**
	rrlD	7.0k	74	35112	20582	**1.7**	**1.9**
	dacB	3.8k	72	14606	16613	**0.9**	**2.2**
	trpA	5.9k	28	3621	8800	**0.4**	**0.6**
							(*pac*)*trp* / (arg)/*trp*
25/09/2000	*holC*	0.7k	97	1855	3344	**0.6**	0.6
P1lysates	*yjfJ*	1.2k	95	3040	4520	**0.7**	0.8
31/07/2000	*pgi*	4.6k	91	7172	15018	**0.5**	0.5
	metB	8.0k	89	161080	15062	**10.7**	12.2
	yihU	2.4k	88	117323	7228	**16.2**	18.6
	hemD	2.0k	86	144318	11261	**12.8**	14.7
	atpG	13.5k	84	34288	13273	**2.6**	3.0
	rfaS	1.4k	82	31040	4384	**7.1**	8.1
	yhjY	1.7k	80	80621	11742	**6.9**	7.8
	zntA	10.8k	78	296984	80542	**3.7**	4.2
	yhfX	3.0k	76	46665	10334	**4.5**	5.2
	rrlD	7.0k	74	56885	26969	**2.1**	2.4
	dacB	3.8k	72	31601	8770	**3.6**	4.1
	trpA	5.9k	28	8402	9604	**0.9**	1.0
01/03/2001	*ygfL*	2.5k	65	5132	3026	**1.7**	
P1lysates	*hycB*	3.1k	61	9389	6976	**1.3**	
31/07/2000	*hemF*	13.5k	55	1509	1434	**1.1**	
	yfaX	1.4k	51	6281	6644	**0.9**	
	yeeA	1.8k	45	6835	7929	**0.9**	
	ydfH	2.2k	35	6803	8233	**0.8**	
30/04/2001	*nadR*	3k	100	178.7	414	**0.4**	
P1lysates	*pstG*	6.7k	25	125.9	218	**0.6**	
31/07/2000	*ybjV*	2.3k	20	285.4	561	**0.5**	
	yleB	4.6k	15	198.8	393	**0.5**	
	ybaV	1.8k	10	222.7	387	**0.6**	
	yaeE	1.2k	5	1084	1880	**0.6**	

A

Dates	Markers	band size on the gel	min on the chrom.	band intensity of P1(arg<>*pac*)	band intensity of P1(-argB)	(*pac*)/trp to (-argB)/trp
03/11/2000	*holC*	0.7k	97	757	992	**1.1**
P1lysates 28/03/2000	*yjfJ*	1.2k	95	446	1024	**0.6**
	pgi	4.6k	91	2082	3331	**0.9**
	metB	8.0k	89	18452	2697	**9.5**
	yihU	2.4k	88	13100	795	**22.8**
	hemD	2.0k	86	22313	1387	**22.2**
	atpG	13.5k	84	5240	5425	**1.3**
	rfaS	1.4k	82	2284	751	**4.2**
	yhjY	1.7k	80	6609	1117	**8.2**
	zntA	10.8k	78	60962	22977	**3.7**
	yhfX	3.0k	76	5656	1850	**4.2**
	rrlD	7.0k	74	14746	8827	**2.3**
	dacB	3.8k	72	3619	1728	**2.9**
	trpA	5.9k	28	1934	2672	**1.0**

B

Note: A) Table shows the ratio of P1(*argB*<>*pac*) DNA to P1 (*-argB*) DNA with the lysate made on 31/7/00. B) Table shows the ratio of *trp*-normalized P1(*argB*<>*pac*) DNA to *trp*-normalized P1 (*-argB*) DNA with the lysate made on 28/03/00. *: since P1(*pac*)DNA was loaded 1.3-fold more than P1(-*argB*)DNA was, the band densities of P1(*-argB*)DNA is multiplied by 1.3. The mean ratio in table A is calculated from 29/08/2000 Southern blot and 25/09/2000 Southern blot.

The densities of bands, as pixel area, were measured. To ensure that the Southern results are proportional to the amount of DNA in each band, the densities of sample bands in the chromosomal ladder and in the ladder of P1 (*argB*<>*pac*) DNA were measured and plotted against the DNA concentration (Fig 4.10). As we can see, most diagrams show that the band densities are proportional to the DNA concentration over a density range from 0 to 1,400K (band density) for chromosomal markers. Most band densities in P1 DNA are also proportional to the DNA concentration. The sole exception is the intensity of *atpG* marker, the reason for this is not clear.

Using the data alone, the ratio of chromosomal to P1(*argB*) packaging was measured to see how markers on chromosomes without *pac* sites are packaged by P1. Both lanes of 6μg and 4μg P1(*argB*) DNA were analyzed and ratios to 2μg chromosomal DNA were calculated (Table 4.5). From Table 4.5, we can see the ratios of chromosomal to P1(*argB*)DNA for most of markers detected are similar, except for *holC* and *trpA*. The reason why the ratio for *holC* is high and *trpA* is low is not clear. This observation shows that most markers on a *pac*-free chromosome are packaged by P1 at similar efficiency.

Table 4.5 Summary of generalized packaging of markers on the chromosome without *pac* sites

marker names	min	6μg band intensity	1μg chro. band intensity	Ratio of 1μg marker /chro.	4μg band intensity	1μg chro. band intensity	Ratio of 1μg marker 4/chr.
holC	96.6	4328	3698	**0.2**	3158	3698	**0.22**
yjfJ	95	10323	12213	**0.13**	5915	12213	**0.13**
pgi	91.2	25958	35971	**0.12**	19272	35971	**0.13**
metB	88.9	26095	45363	**0.1**	19177	45363	**0.1**
yihU	87.7	12125	25133	**0.08**	9294	25133	**0.1**
hemD	85.6	21954	30779	**0.12**	14339	30779	**0.13**
atpG	84.4	22560	29393	**0.13**	17796	29393	**0.15**
rfaS	81.7	8321	12266	**0.12**	5499	12266	**0.1**
yhjY	80	22983	29617	**0.13**	14943	29617	**0.13**
zntA	77.7	129084	156388	**0.13**	90577	156388	**0.15**
yhfX	75.6	16458	26999	**0.1**	13058	26999	**0.13**
rrlD	73.8	43200	52861	**0.13**	30295	52861	**0.15**
dacB	71.7	14851	21526	**0.12**	11080	21526	**0.13**
trpA	28.3	14287	57247	**0.03**	10512	57247	**0.05**

The densities of 26 markers in total were then measured and the ratio of P1 (arg<>*pac*) DNA to P1 (-*argB*) DNA for each marker was calculated and plotted against the chromosomal min unit map (Table 4.4, Fig. 4.12). To ensure that the data are repeatable, another lysate made at a different time from the most frequently used lysate was tested as well (Table 4.4B). Since the concentration of P1 DNA purified from this lysate was not recorded, the ratio between P1(*argB*<>*pac*) and P1(-*argB*) for each marker was normalized by setting the *trp* intensity ratio equal to 1.0. As we can see, Southern blot data using P1 DNA from different lysates showed similar patterns of packaging.

It is clear that the chromosomal *pac* site, which is located at 89 min, is directional, since the packaging only of markers to one side of *pac* has been increased, while that of markers to the other side was in fact reduced. This observation is consistent with the finding that the end destined to be packaged is protected from cellular nucleases, while the end not destined to be packaged will be rapidly degraded by host nucleases (Sternberg and Coulby, 1987a). In this experiment, we showed that the reduction of the packaging spans at least 30min, from the *pgi* marker, which is located at 91min and gives a ratio of P1(*argB*<>*pac*) to P1 (-*argB*) equal to 0.36, to the *pstG* marker, which is located at 25min and gives a ratio of about 0.6. This could, at least, partially be accounted for degradation of the not-packaged end by nucleases. The deep trough for *atp*G is puzzling. Further work might include the measurement of the packaging of markers very close to atpG to see whether the effect is somehow marker specific or whether atpG is in a genuine region of reduced packaging

In Sternberg's work (Sternberg and Coulby, 1987a), it was found that for markers within 10min (5 P1 "headful") of *pac*, the increase in transduction frequency is relatively independent of distance from *pac*, while for markers that are located 10 to 20min from *pac*, the increase in transduction frequency decreases with distance from *pac*; there is no increased transduction of markers further than 20min from *pac*. However, in my work (Fig 4.12), the packaging of markers up to 30min from *pac* was increased from *pac* at 89min to *hemF*, 55min with the effect diminisings with distance from *pac*. The effect of *pac* on packaging of the chromosome thus appears to extend about 10min further than that observed for transduction frequency. From the data in Table 4.1, we can see that the transduction frequency of *metB* has been increased a hundred fold by the *pac* site although the packaging of the *metB* gene is only increased by about 10-fold. This observation could indicate that less abortively transduced DNA is formed from the *pac* containing strain than from the wild type strain.

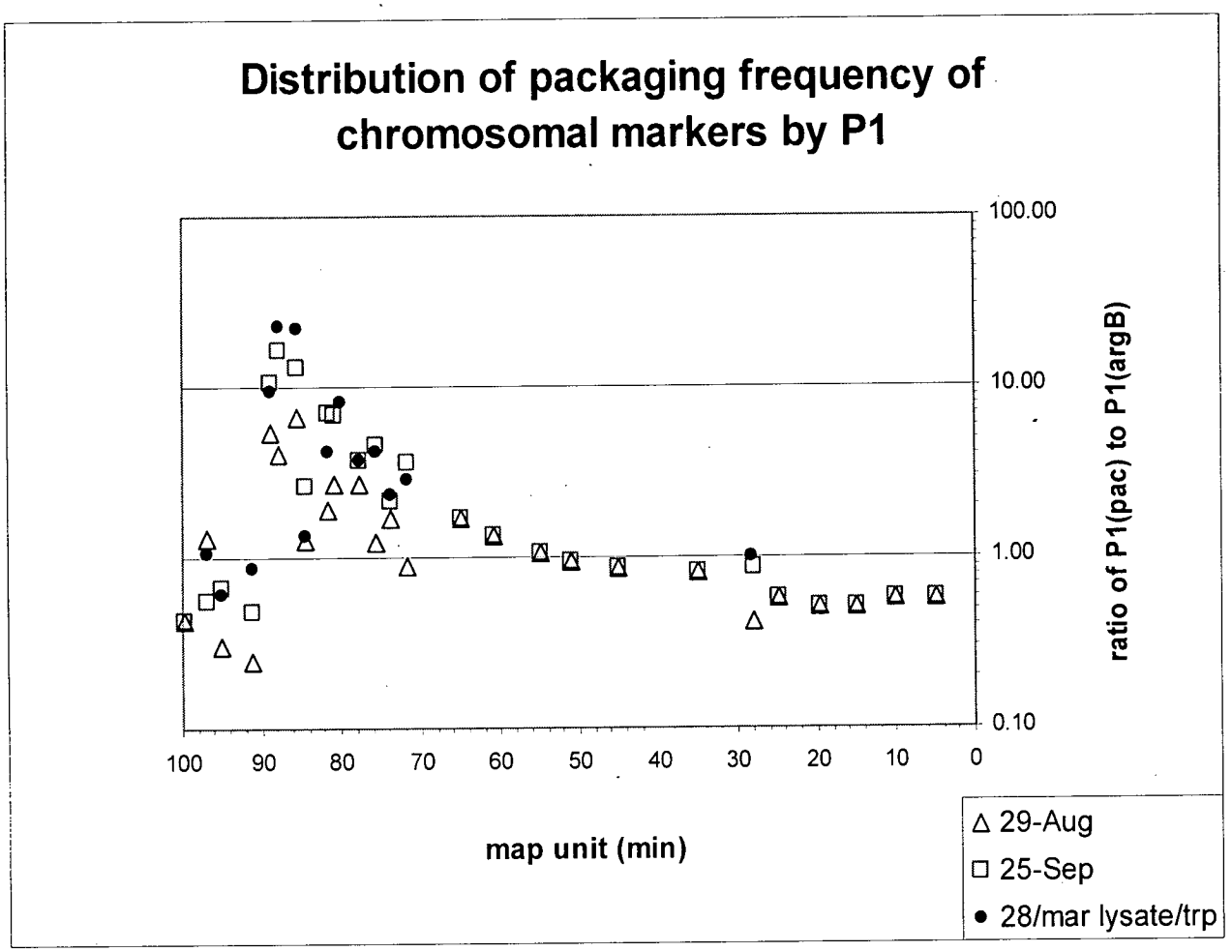


Fig 4.12 Relative packaging of markers from MG 1655 (-arg) and MG1655 (arg<>*pac*). The density ratio of P1 (*argB*<>*pac*) DNA to P1 (*-argB*) DNA for each marker was calculated and plotted against the map position. The detailed description is in the text. For lysates made on 28th/03, 14 markers were measured and the density ratio was normalized with respect to *trp* (see the text for detail).

4.2.2. *pac* at other locations on the chromosome

As mentioned above, in order to increase the fraction of transducing DNA, a *pac* site was put on the chromosome. As expected, this results in increased transducing DNA containing markers close to the *pac* site. We then went on to determine the effects of putting multiple *pac* sites on the chromosome.

4.2.2.1. Construction of strains containing *pac* at other locations

Four more *pac*-containing strains were made by an improved homologous recombination method (Merlin *et al.* 2002). To introduce 4 further *pac* sites at locations 20 min apart, I designed a new multiple cloning site to replace the original limited multi-restriction site on pKO3 (Fig. 4.13). Several 8 base-pair restriction sites were included in the new linker so that the new multi-restriction sites could be used for cloning most DNA sequences desired. Two blunt restriction sites, *Sma*I and *Swa*I, are in the middle of the linker and were used to insert the pre-blunted P1 EcoRI-20 fragment and the FRT-KAN cassette, respectively. The orientations of *pac* and the Kanamycin gene are opposite. Each flanking region of the *pac*-KAN cassette has 3 cloning sites (Fig. 4.14). The features of this multi-cloning site allow us to: a) insert the *pac*-FRT-KAN cassette anywhere on the chromosome by changing only the flanking gene arms without disturbing the *pac* fragment and the FRT-KAN cassette. b) we can control the orientation of *pac* on the chromosome by placing flanking arms appropriately.

ctgca GGGATCCTTAATTAAGAGCTCCCCGGGATTTAAATGCTAGCGCGGCCGCGGCGCGCCG *tcgac*
g ACGTCCCTAGGAATTAATTCTCGAGGGGCCCTAAATTTACGATCGCGCCGGCGCCGCGCGGCAGCT *g*

PstI BamHI PacI SacI SmaI SwaI NheI NotI AscI SalI

Fig 4.13 The new polylinker for the reconstruction of the multi-cloning site on pKO3

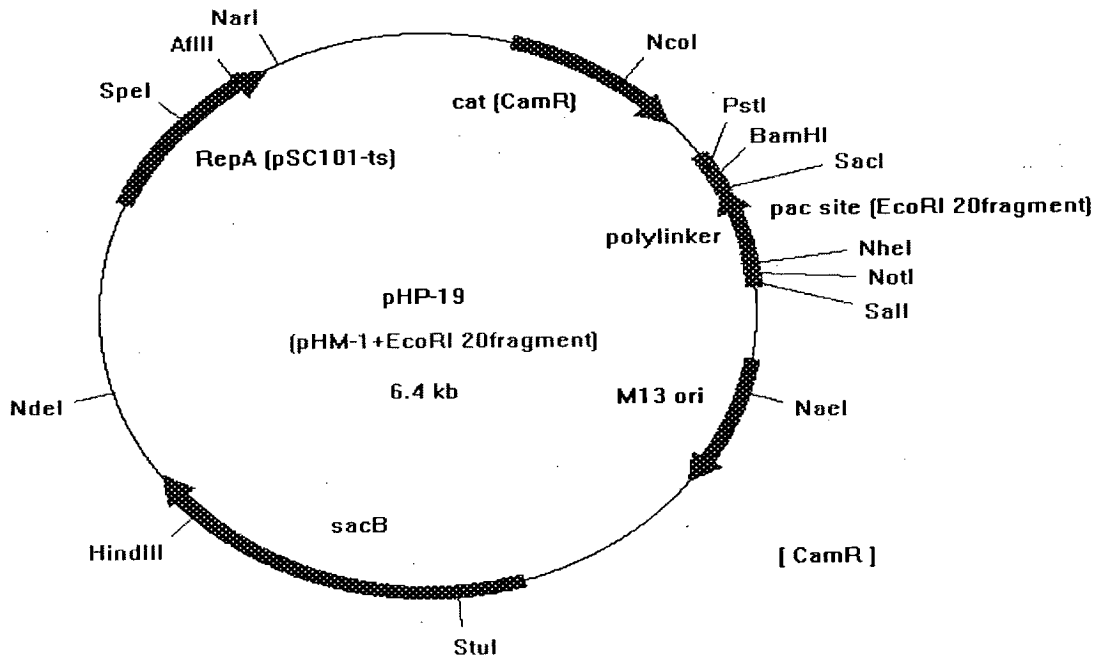
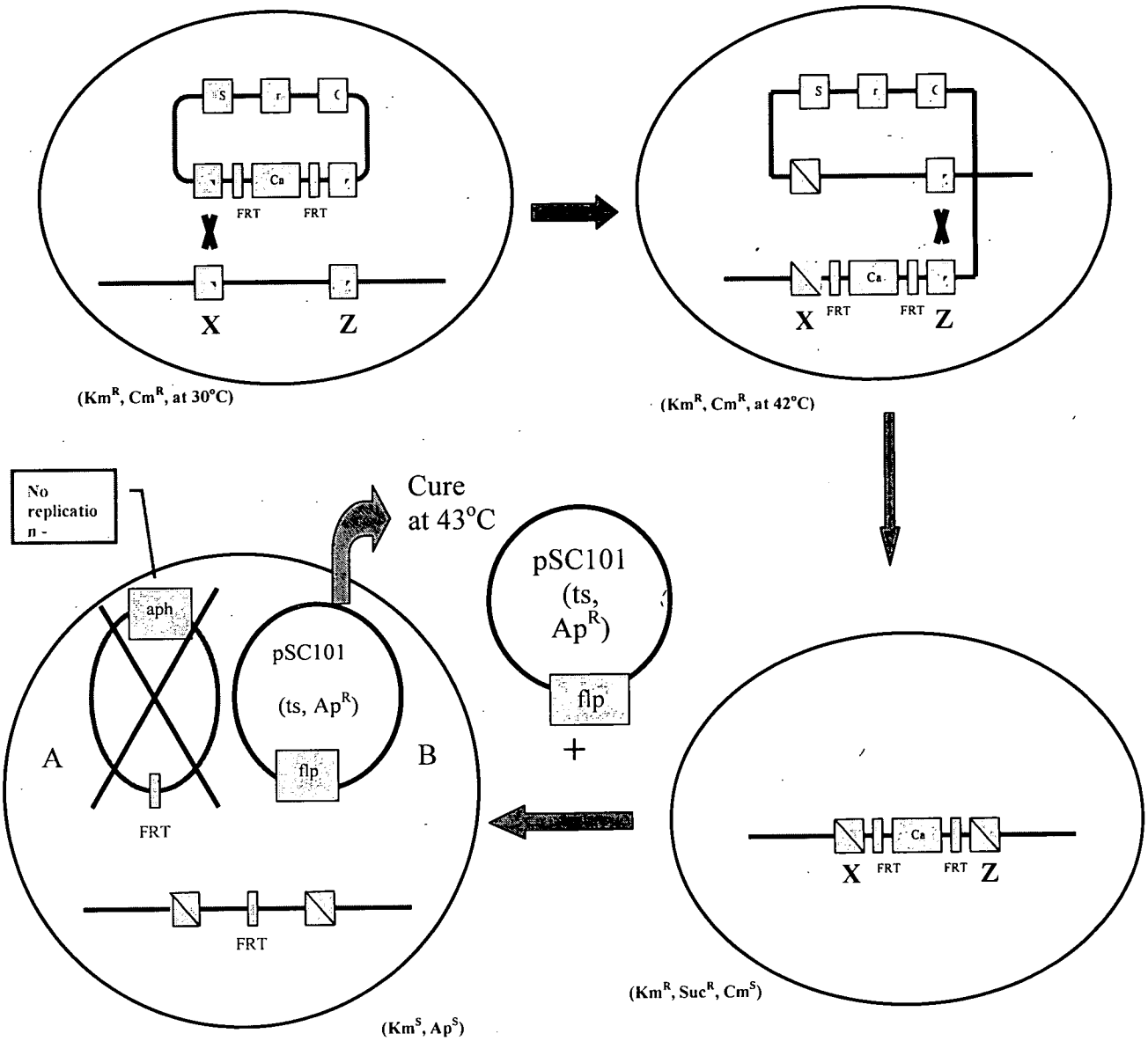
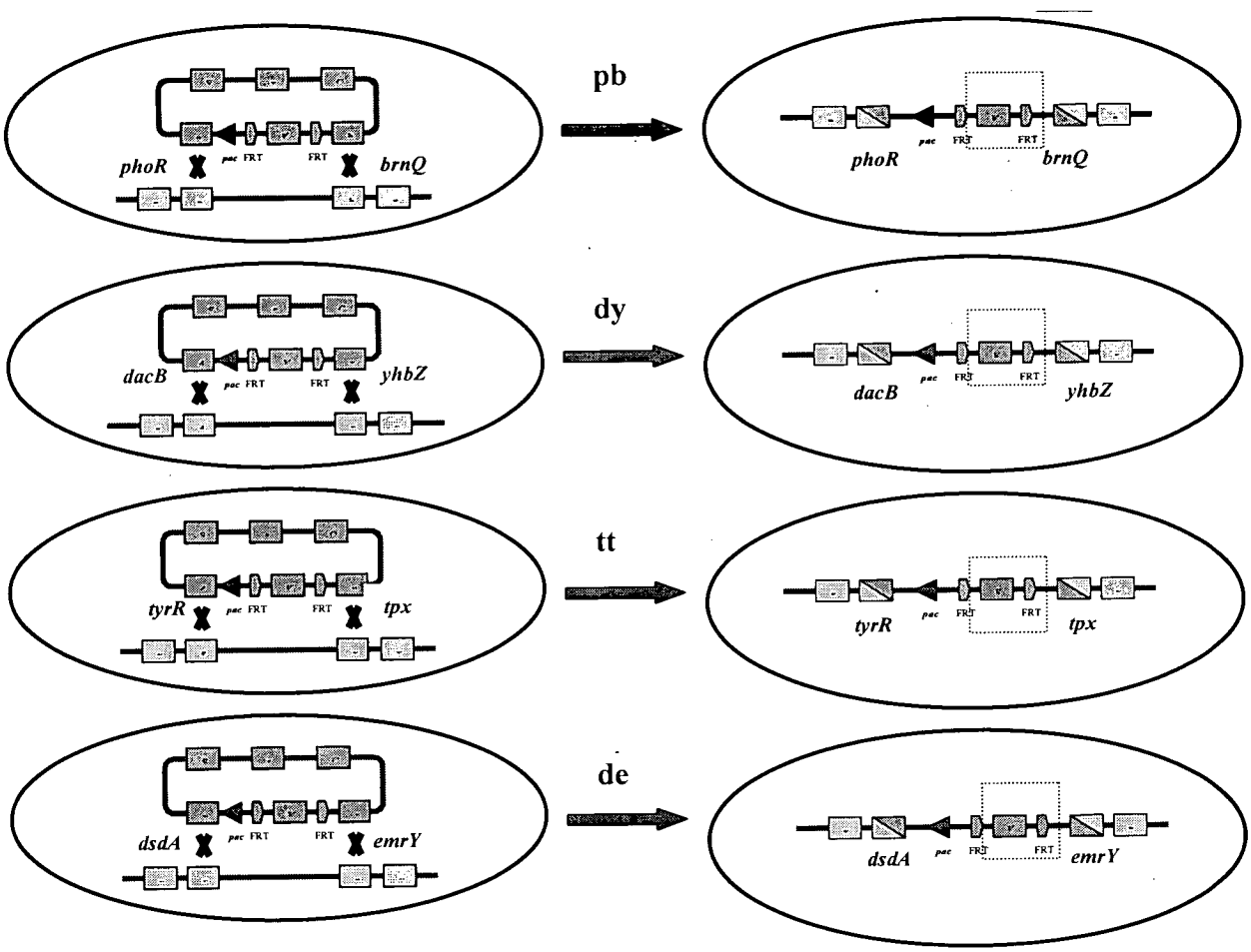


Fig 4.14 The new pKO3-based *pac*-inserting plasmid, pHP-19

Another feature of the new replacement plasmid is the FRT-KAN cassette. In this cassette, the gene specifying Kanamycin-resistance (the *aph* gene, Merlin, *et al.*, 2002) gene is flanked by two FRT sites from yeast in direct orientation. This allows removal of the cassette by the Flp site-specific recombinase provided in *trans* on pCP20 (Fig. 4.15) (Merlin, *et al.*, 2002). This allows selection and screening of the integrated strains by taking advantage of the *sacB* gene as described above. The FRT-KAN cassette next to *pac* is integrated into the chromosome along with *pac*, and renders the strain resistant to Kanamycin. This allows us to select the *pac*-integrated and plasmid cured strain by selecting for Kanamycin resistance and then screening for loss of chloramphenicol resistance. It also facilitates the insertion of the *pac* site between ORFs without interrupting any genes. Once the *pac*-containing Kanamycin resistant strains are obtained, then the *aph* gene can be removed from cells by using the easily cured temperature sensitive vector (pCP20) containing the Flp site-specific recombinase gene. This feature allowed us to create multi-*pac* strains using P1 transduction to introduce each of the constructs successively from different single replacement strains.


FRT
FRT
X
Z
(Km^R, Cm^R, at 30°C)
(Km^R, Cm^R, at 42°C)
X
FRT
FRT
Z
No replicatio n -
Cure at 43°C
pSC101 (ts, Ap^R)
flp
+
aph
pSC101
(ts, Ap^R)
flp
A
B
FRT
FRT
(Km^S, Ap^S)
(Km^R, Suc^R, Cm^S)
X
FRT
FRT
Z

A



B

Fig 4.15 Diagram of the improved protocol for placing the *pac* site onto the chromosome and the locations where the *pac*-FRT-KAN cassetteswere inserted to. **A**) the improved procedure to insert *pac* sites into the chromosome (see Materials and Methods). **B**) the locations where the *pac*-FRT-KAN cassettes were put.

4.2.2.2. Constructing and testing of more *pac*-containing strains

First of all, 4 further strains with single *pac* sites were constructed using the method described in the above section. Strains MG(1-pb), MG(1-de), MG(1-dy) and MG(1-tt) each contain a single *pac* site located at 9 min, between gene *phoR* and *brnQ*, 53.4 min, between gene *dsdA* and *emrY*, 71.7 min, between gene *dacB* and *yhbZ* and 29.9 min, between *tyrR* and *tpX* respectively (Fig 4.16). The constructed strains were verified immediately with PCR.

P1 transduction was used once again to test if the effect on transduction frequency by *pac* on the chromosome is location-dependent. Markers close to each *pac* were tested. All tested markers were within 2 min of *pac*, so that they were expected to be packaged in the same or an adjacent "headful", relative to *pac*. The transduction frequency of each of the markers is greatly increased as expected and listed in Table 4.5. We can conclude that the effect of *pac* on packaging of transducing DNA is likely independent of the location of *pac* on the chromosome. The significant increase of transduction frequencies of markers close to the *pac* site also indicates a functional *pac* site located at the corresponding position on the chromosome (Table 4.6).

Once single-*pac* containing strains were constructed, P1 transduction was used to construct multiple-*pac*-site containing strains (Fig 4.17). Kanamycin resistant transductants were selected after each P1 transduction. The *aph* gene was removed by FRT site-specific recombination before further P1 transductions were carried out in order to put more *pac* sites into the same chromosome. PCR and transductions was used to verify the constructed 5-*pac* containing strain (Fig 4.18, Table 4.7).

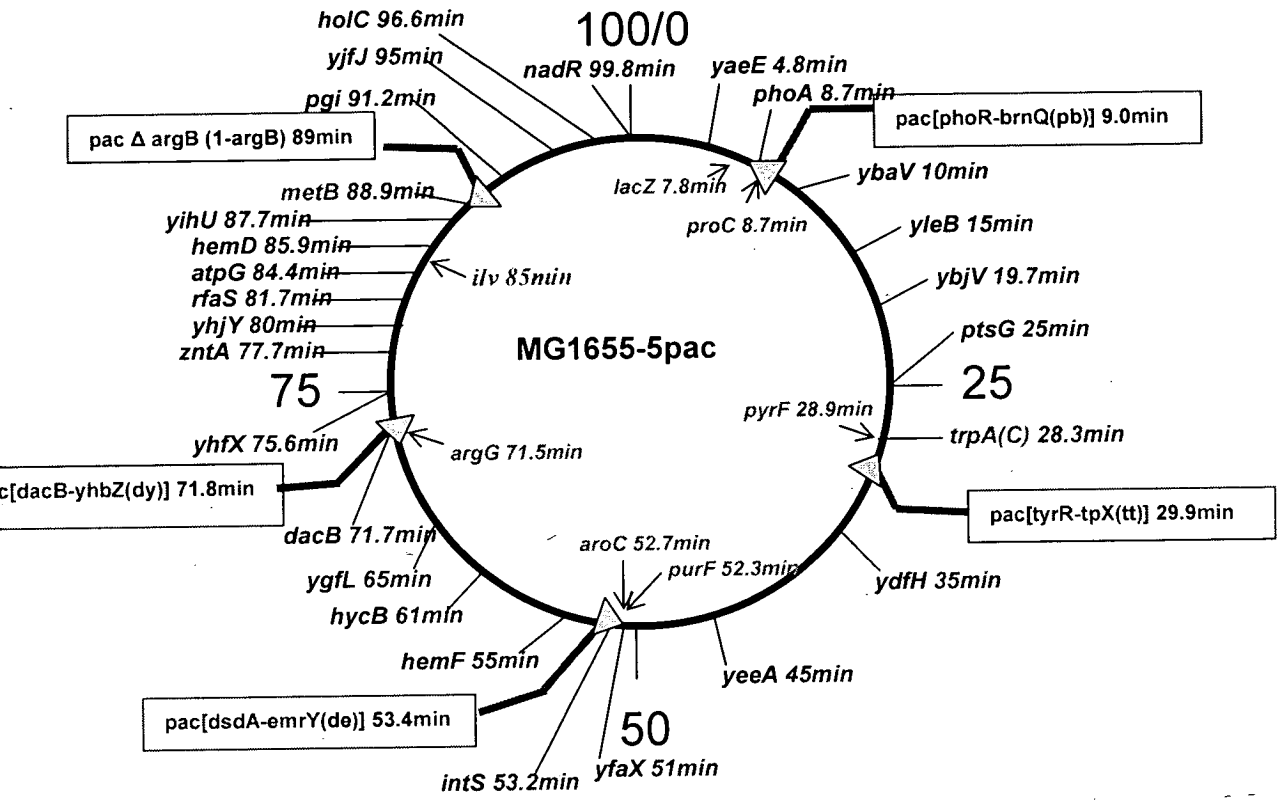


Fig 4.16 The locations of the *pac* sites on the chromosome and markers along the entire chromosome for the probes of Southern blot. The triangles stand for *pac* sites on the chromosome and are pointing as packaging will proceed. The genes outside the ring followed by their position (min) on the chromosome are for Southern probes. The markers inside ring were used for transduction.

Table 4.6 Transductions from different *pac* containing strains

Recipients	MM303					
Selection markers	*metB* (89 min)		*lac* (9 min)		*trpA* (28 min)	
Strains of P1	P1(MG)	P1(1-arg)	P1(MG)	P1(1-pb)	P1(MG)	P1(1-tt)
Titer of P1(pfu/ml)	$5x10^8$	$3x10^8$	$5x10^8$	$2.5x10^8$	$5x10^8$	$1.6x10^8$
No. of colonies	**145**	**77300**	**197**	**55300**	**110**	**5900**
Multiplied no./ml	**1450**	**773000**	**1970**	**553000**	**1100**	**59000**
No./phage	$\mathbf{2.9x10^{-6}}$	$\mathbf{2.6x10^{-3}}$	$\mathbf{3.9x10^{-6}}$	$\mathbf{2.2x10^{-3}}$	$\mathbf{2.2x10^{-6}}$	$\mathbf{3.7x10^{-4}}$
Ratio to P1(MG)		**897**		**564**		**168**

Recipients	MM38		AB325	
Selection markers	*argG* (71 min)		*Ade* (53 min)	
Strains of P1	P1(MG)	P1(1-dy)	P1(MG)	P1(1-de)
Titer of P1(pfu/ml)	$2x10^9$	$1x10^9$	$5x10^8$	$2x10^8$
No. of colonies	**127**	**83200**	**33**	**11400**
Multiplied no./ml	**1270**	**832000**	**330**	**113000**
No./phage	$\mathbf{6.4x10^{-7}}$	$\mathbf{8.3x10^{-4}}$	$\mathbf{6.6x10^{-7}}$	$\mathbf{4.7x10^{-4}}$
Ratio to P1(MG)		**1297**		**712**

Note: P1 lysates were made from different single *pac*-containing strains. The names of different P1 lysates correspond to the names of strains (see Table 2.1). The locations of the *pac* sites on the chromosome refer to Fig 4.8. Recipients (MM303, MM18 and AB325) were grown up to OD_{600} = 1-1.5. The lysates of P1(1-arg), P1(1-pb), P1(1-de), P1(1-dy) and P1(1-tt) were diluted 100-fold respectively. 100 µl of corresponding diluted P1 lysates as well as P1(MG) lysates which was not diluted were mixed with 100 µl of recipients and the mixture was incubated at 37°C for 15min. 2.5ml of top water agar was then added to the mixture and poured onto the corresponding selection plates. The plates were incubated at 37°C overnight.

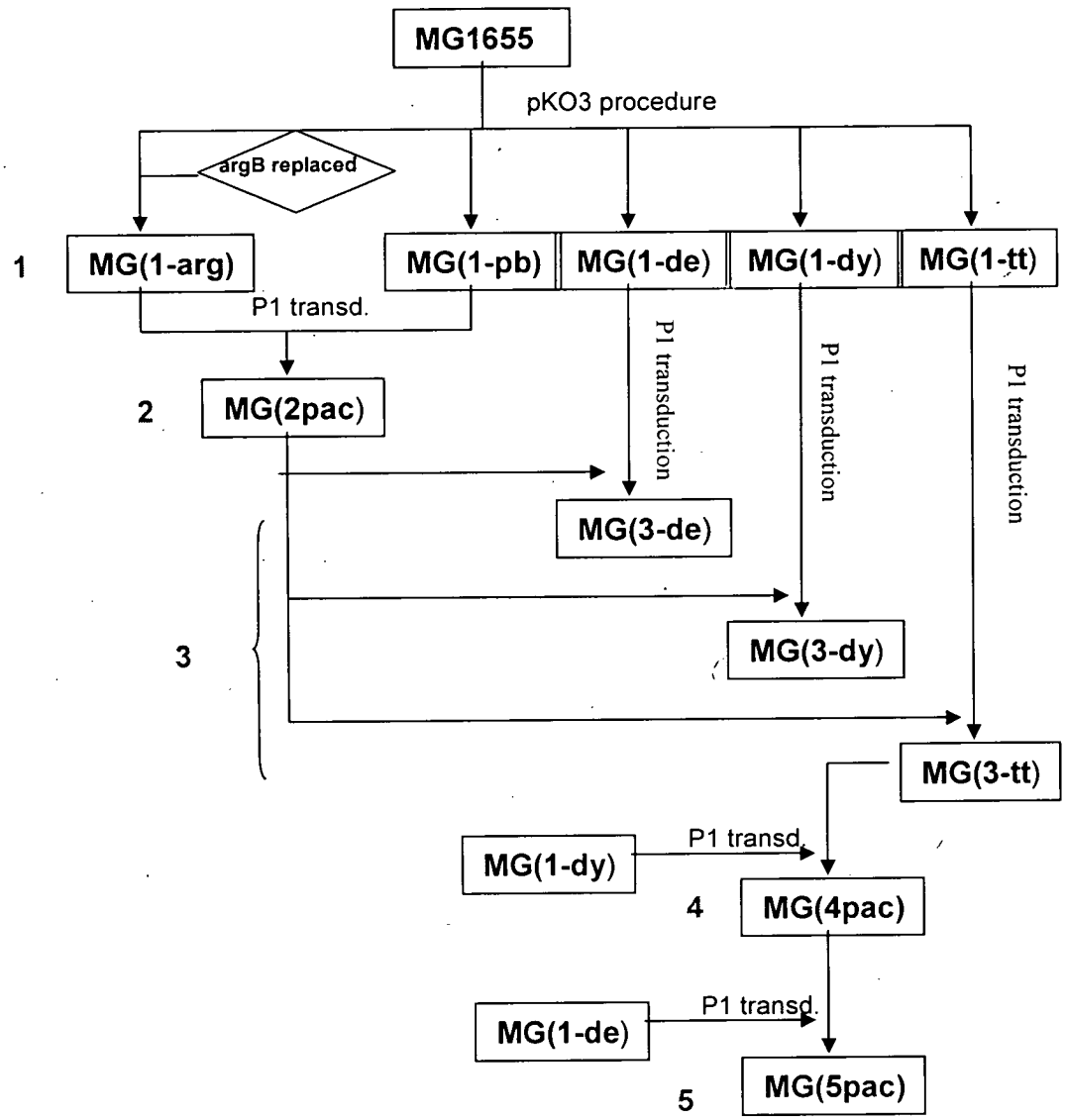


Fig 4.17 Flow sheet of construction of multi-pac-site strains. 5 single-pac-site strains were constructed using pKO3 procedure and all multi-pac strains were constructed by P1 transduction. All strains containing more than 2 *pac* sites are derived from the same MG (2pac) strain. See details in the text.

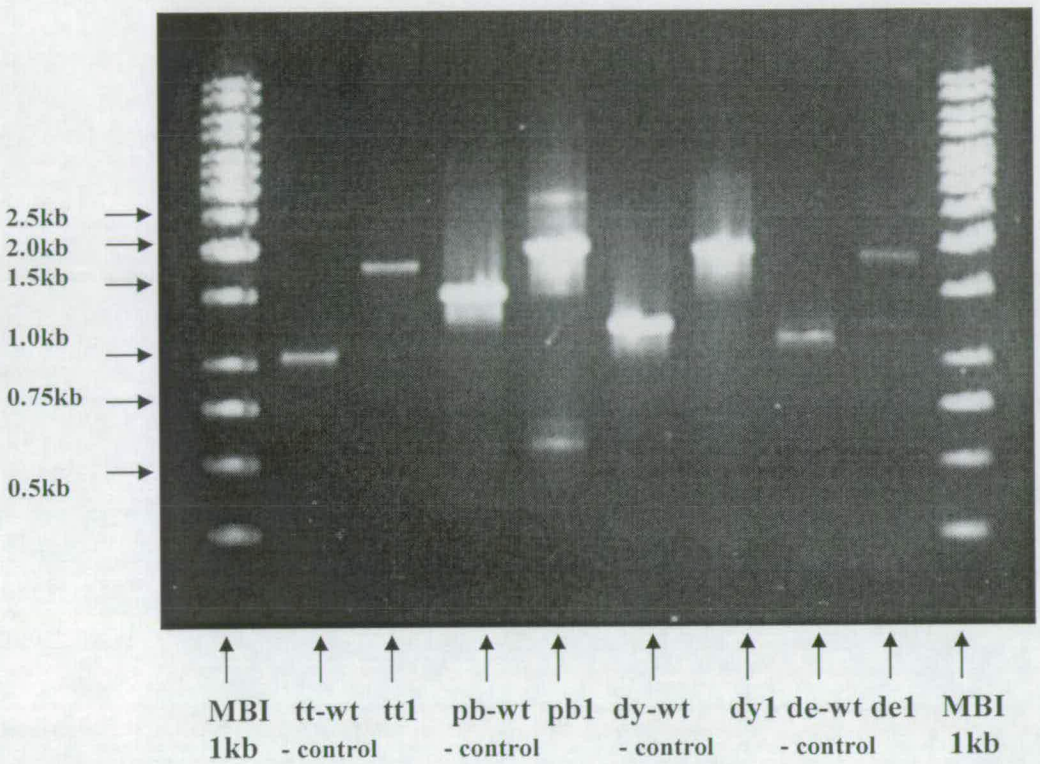
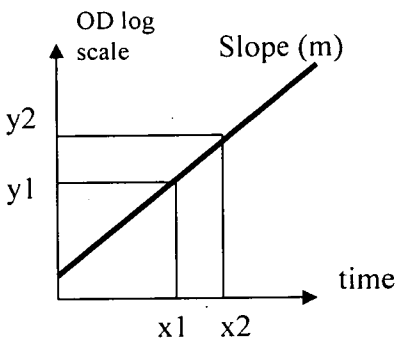


Fig 4.18 Verification of *pac* sites in MG1655 (5pac, kan$^-$). de1, dy1, pb1 and tt1 correspond to the position on the chromosome where *pac* is located. e.g. **de1** stands for the *pac* site inserted in between *dsdA* and *emrY* gene (see Materials and Methods). de-wt – control, dy-wt – control, pb-wt – control and tt-wt – control are *pac*-free controls and all were amplified from MG1655 but with different sets of primers synthesized for insertion of *pac* into the above corresponding locations. The strain I tested here is MG1655 (5pac, kan$^-$), which only contains P1 EcoRI-20 fragment (650bp) on the chromosome while the FRT-KAN cassette has been removed. The 5th *pac* site is the *pac* which replaces *argB* gene. Since all the multi-*pac* strains are based on MG1655 (2pac) and constructed by P1 transduction, and MG1655 (2pac) was derived from MG1655 (1-*argB*) by inserting an extra *pac* in between *phoR* and *brnQ* gene using the above improved pKO3 procedure (See the following Fig 4.15.1), the verification of this *pac* site can be seen in Fig 4.5. For de: the expected size of the band is 1.96 kb, while the minus control is 1.17 kb; for dy: the expected size of the band is 2.01 kb, while the minus control is 1.24 kb; for tt: the expected size of the band is 1.86 kb, while the minus control is 1.09 kb and for pb: the expected size of the band is 1.95 kb, while the minus control is 1.56 kb. The unexpected bands in lanes of pb1 and de1 probably stand for unspecifically amplified products.

4.3. Multi-*pac* sites on the chromosome

4.3.1. Growth curves of different *pac*-containing strains

Since the chromosomes of cells have been altered, growth curves for all strains were measured to ensure that the differences in the subsequent experiments are not due to the differences in growth rate of those strains. The doubling time was calculated according to the formula below. The growth curves were measured as described in Materials and Methods. In order to plot the graph, the ODs were multiplied by the dilution factor and plotted as a continuous growth curve. Fig 4.19 shows growth curves and doubling times of some representative strains. Slight differences were found in growth rates of these strains, over a range of about 21-23 min in LB. Since the 5 *pac* strain grew no more slowly than the slowest single *pac* strains (de or tt), multiple *pac* sites cannot be a cause of slowed growth. It is more likely that the particular insertions (de and tt) somehow reduced growth rate.



OD *v*.s. Time

$\mathbf{y = p \cdot e^{(m.x)}}$ (y = OD; x = time; p & m are constants)

⇨ $\mathbf{\ln(y) = \ln(p \cdot e^{(m.x)})}$
⇨ $\mathbf{\ln(y) = \ln(p) + \ln(e^{(m.x)})}$
⇨ $\mathbf{\ln(y) = \ln(p) + m.x}$

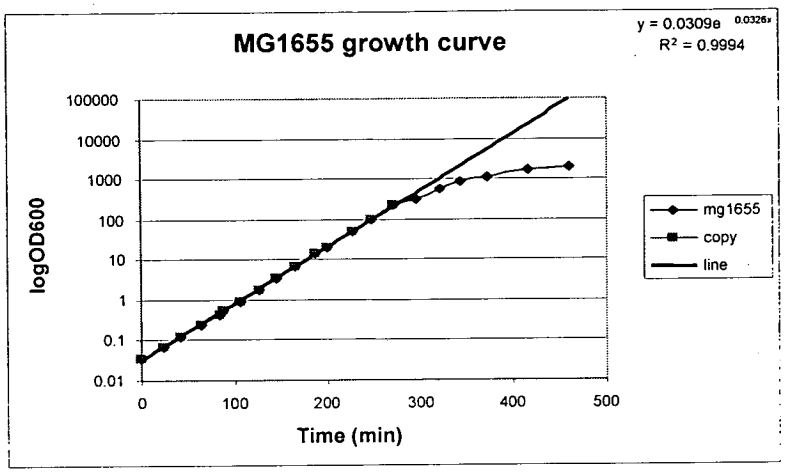
There is a linear relationship between x and log(y)

$\mathbf{m = \Delta \ln(y)/\Delta x = \ln(y2) - \ln(y1)/\Delta x = \ln(y2/y1)/\Delta x}$

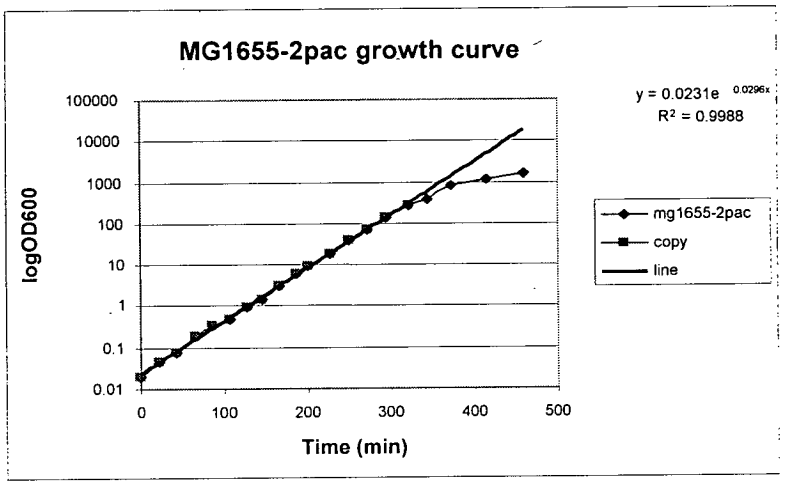
⇨ $\mathbf{\Delta x = \ln(y2/y1)/m}$ (m, a constant number provided in the equation)

Since, y2/y1 = 2, when culture is doubled

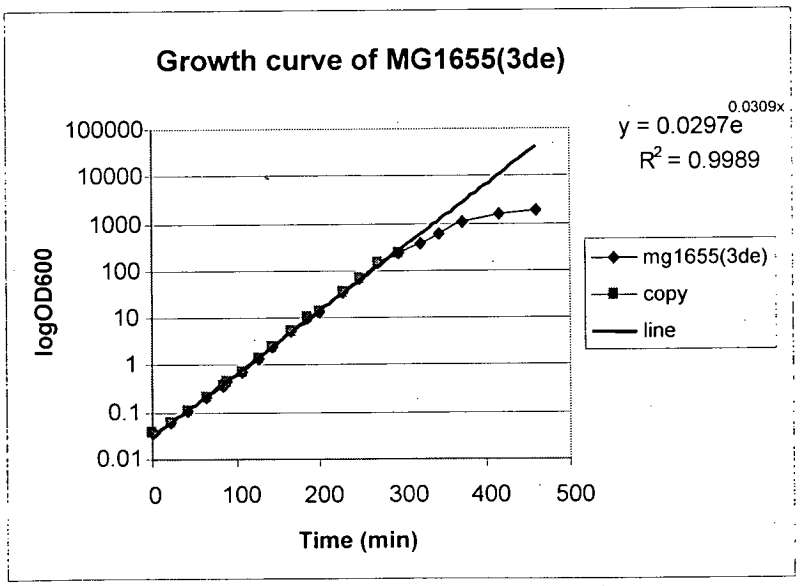
Doubling time = Δ x = ln2/m (a constant number)



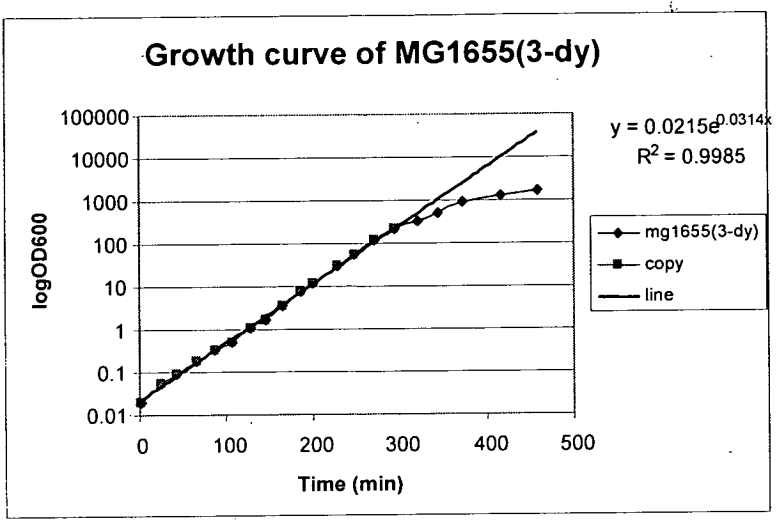
Doubling time = 21.3



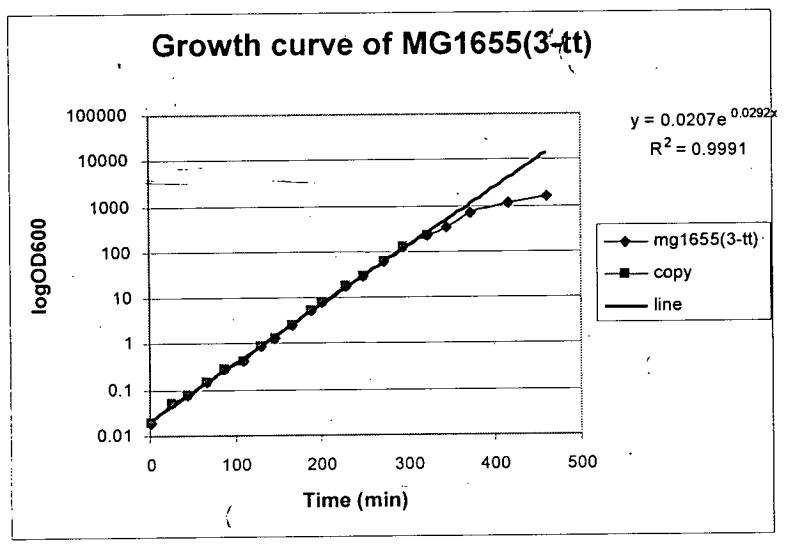
Doubling time = 23.4



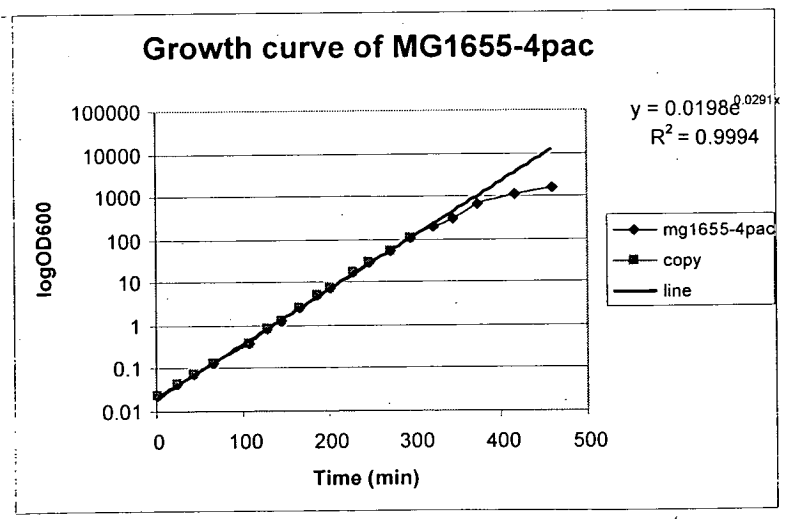
Doubling time = 22.4



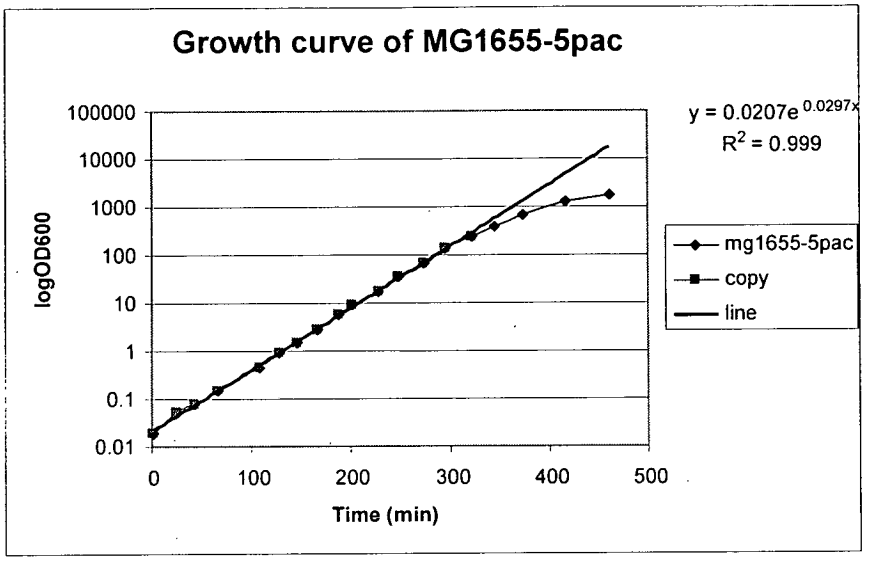
Doubling time = 22.1



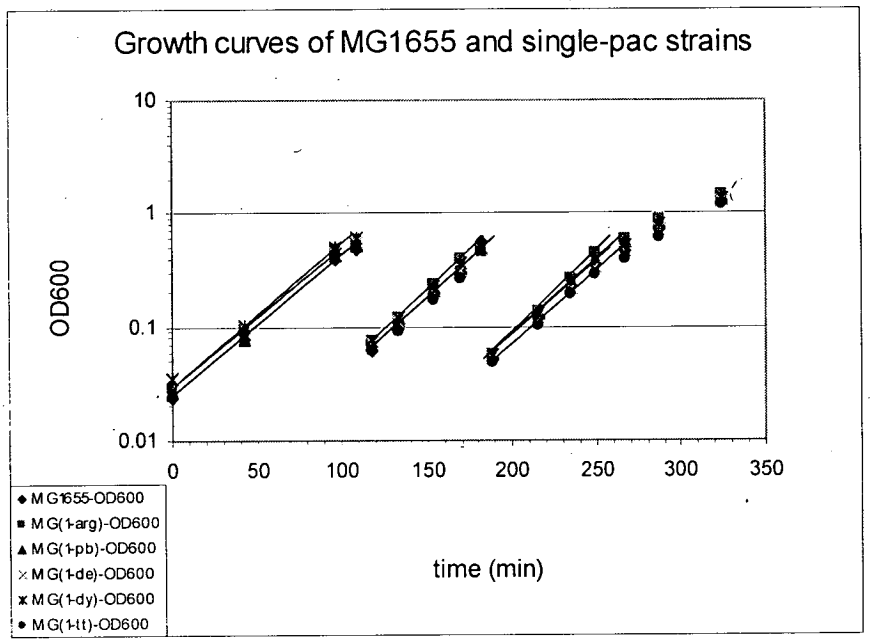
Doubling time = 23.7



Doubling time = 23.8



Doubling time = 23.3



	MG1655	MG(1-arg)	MG(1-pb)	MG(1-de)	MG(1-dy)	MG(1-tt)
Doubling Time	21.2 min	21.6 min	20.8 min	23.5 min	22.5 min	23.5 min

Fig 4.19 Growth curves and doubling times of MG1655 and *pac*-containing strains. Except for growth curves of single *pac* containing strains, growth curves and doubling times were plotted and calculated using the formula above and described in the text. In order to draw a trendline using Excel for calculation of doubling times, a copy of exponential range of the growth curve was made and plotted as squares.

4.3.2. Effect of multiple *pac* sites on transduction frequency

We already know that one *pac* site on the chromosome can increase the transduction frequencies of downstream markers. However, what would happen if there is more than one *pac* on the chromosome: are the effects on the transduction frequencies of a particular marker by more than one *pac* additive or do the effects counteract each other? We know that *pac* has orientation and that DNA is packaged into the capsid unidirectionally. Transduction experiments have shown clearly that the transduction frequencies of markers to one side of *pac* are significantly increased, while the transduction frequencies of markers to the other side of *pac* are not greatly affected. However, Sternberg and Coulby (1987a) also showed that the end not destined to be packaged into the P1 head will be quickly degraded by host nucleases, which means that instead of being increased, the transduction frequencies of markers close to and on the opposite side of *pac* will be diminished. To test how multiple *pacs* affect the transduction frequency of genes, transduction was carried out using four different P1 liquid (1) lysates, P1 (MG), P1 (2pac), P1 (3-de) and P1(5pac), which were made from MG1655, MG1655(1-arg), MG1655(3-de) and MG1655 (5pac) respectively. Only one recipient, MM303, was used.

We also hoped to create a strain that would be particularly useful as a transduction donor. If the total number of transducing phage produced in a lysate is greatly increased by having multiple packaging sites in the chromosome, it is possible that transduction frequencies could be sufficiently increased, at least for some markers, to enable scoring for transduction amongst transduced populations where selection is difficult.

As mentioned in Materials and Methods, three types of P1 lysates were used in this work: P1 plate lysates, P1 liquid lysates, P1 single cycle lysates. Usually, P1 plate lysates, which are made by overnight multiple-cycle infection, are used for P1 transducing stocks, since they usually have the highest titer. P1 single cycle lysates are made by adding extra wash and resuspension steps in order to promote synchronous infection and to prevent multiple re-infections. P1 liquid lysates are usually used for making large scale lysates for phage concentration and DNA preps. These result from one cycle of P1 infection, but without the use of special p*recA*utions to guarantee synchronous infection. In the work presented in this section, P1 liquid lysates were used in transduction.

The transduction frequencies for different markers obtained with various P1 lysates are listed in Table 4.7. The ratio of transduction frequencies obtained from P1 multi-*pac* lysates to that of a P1(MG1655) lysate was also calculated and plotted in order to determine the effects of different numbers of pac*s* on transduction of particular markers (Fig. 4.20). We can see from the table that, as was the case for the single-*pac* lysates, the transduction frequencies of markers which are located close and to one side of *pac* has been greatly increased, no matter where the marker is located on the chromosome. However, measurement of transduction frequencies is not sensitive enough to show the interaction between *pacs* on the multi-*pac* donor. Note, however, that the transduction frequencies of *ilv* and *hisG* in P1(5pac) lysates are less than that in P1(2pac) and P1(3de) lysates suggesting that markers well behind a *pac* site may suffer a reduction in transduction. However, we cannot rule out the possibility that a general decrease in transduction of everything occurs when there are as many as 5 *pac* sites. Because the number of markers that can be used for transduction is limited and because of the complexity of the transduction process, Southern blots were again used to examine, in detail, packaging of transducing DNA from multi-*pac* donors.

Table 4.7 A) Transduction frequencies for selected markers using various P1 lysates

		P1(MG)	P1(2pac)	P1(3-de)	P1(5pac)
pac locations (min)			89, 9	89, 9, 53	89,9,30,53,72
Titer (pfu/ml)		3×10^9	4×10^8	1×10^9	5×10^8
markers		Recipient strain: **MM303**			
argH 89.5min	No. /plate	590	2	3	0
	No. /ml	5900	20	30	0
	No./phage	2.0×10^{-6}			
metB 88.9min	No. /plate	686	4556	4988	3308
	No. /ml	6860	4556000	4988000	3308000
	No./phage	2.3×10^{-6}	1.1×10^{-2}	5.0×10^{-3}	6.6×10^{-3}
ilv 85min	No. /plate	608	320	1099	247
	No. /ml	60800	320000	1099000	247000
	No./phage	2.0×10^{-5}	8×10^{-4}	1.1×10^{-3}	4.9×10^{-4}
hisG 45min	No. /plate	153	210	89	82
	No. /ml	1530	2100	89000	8200
	No./phage	5.1×10^{-7}	5.3×10^{-6}	8.9×10^{-5}	1.6×10^{-5}
trpA 28.3min	No. /plate	114	343	562	1182
	No. /ml	1140	3430	5620	1182000
	No./phage	3.8×10^{-7}	1.1×10^{-6}	1.9×10^{-6}	2.4×10^{-3}

Note: MM303 was grown up to OD_{600}=1-1.5. P1(MG) lysate was diluted 10-fold only for *ilv* transduction; P1(2pac) lysate was diluted 100-fold for *metB,* and *ilv* transductions; P1(3-de) lysate was diluted 100-fold for *metB*, *ilv* and *hisG* transductions and P1(5pac) lysate was diluted 100-fold for all transductions except for hisG transduction, where 10µl original lysate was used. 100µl of diluted lysates were mixed with 100 µl of recipients and then the mixture was incubated at 37°C for 15min. 2.5 ml of top water agar was added to the mixture and poured onto the corresponding selection plates and the plates were incubated at 37°C overnight. No./ml in the table refers to the number of transductants in 1ml of original lysate.

Table 4.7 B) MM38 was grown up to OD_{600}=1-1.5. P1(5pac) lysate was diluted 100-fold

		P1(MG)	P1(2pac)	P1(3-de)	P1(5pac)
pac locations (min)			**89, 9**	**89, 9, 53**	**89,9,30,53,72**
Titer (pfu/ml)		**3×10^9**	**4×10^8**	**1×10^9**	**5×10^8**
markers		Recipient strain: **MM38**			
argG 71.5min	No. /plate	**110**	**253**	**857**	**446**
	No. /ml	**1100**	**25300**	**85700**	**446000**
	No./phage	**3.7×10^{-7}**	**6.3×10^{-5}**	**8.6×10^{-5}**	**8.9×10^{-4}**

Note:100µl of P1(MG) lysate and diluted P1(5pac) lysate and 10µl of original P1(2pac) and P1(3-de) lysates were mixed with 100 µl of recipients and then the mixture was incubated at 37°C for 15min. 2.5 ml of top water agar was added to the mixture and poured onto the corresponding selection plates and the plates were incubated at 37°C overnight. No./ml in the table refers to the number of transductants in 1ml of original lysate.

Table 4.7 C) MG1655-Δlac was grown up to OD_{600}=1-1.5

		P1(MG)	P1(2pac)	P1(3-de)	P1(5pac)
pac locations (min)			**89, 9**	**89, 9, 53**	**89,9,30,53,72**
Titer (pfu/ml)		**3×10^9**	**4×10^8**	**1×10^9**	**5×10^8**
markers		Recipient strain: **MG1655-Δlac**			
lac 7.8min	No. /plate	**668**	**249**	**430**	**278**
	No. /ml	**66800**	**2490000**	**4300000**	**2780000**
	No./phage	**2.2×10^{-5}**	**6.2×10^{-3}**	**4.3×10^{-3}**	**5.6×10^{-3}**

Note: P1(2pac) lysate, P1(3-de) lysate and P1(5pac) lysate was diluted 100-fold before mixing with cells. 10µl of samples were mixed with 100 µl of recipients and then the mixture was incubated at 37°C for 15min. 2.5 ml of top water agar was added to the mixture and poured onto the corresponding selection plates and the plates were incubated at 37°C overnight. No./ml in the table refers to the number of transductants in 1ml of original lysate. Note that since growth conditions are different for different recipients, widely different transduction frequencies for the same marker were obtained with different recipients.

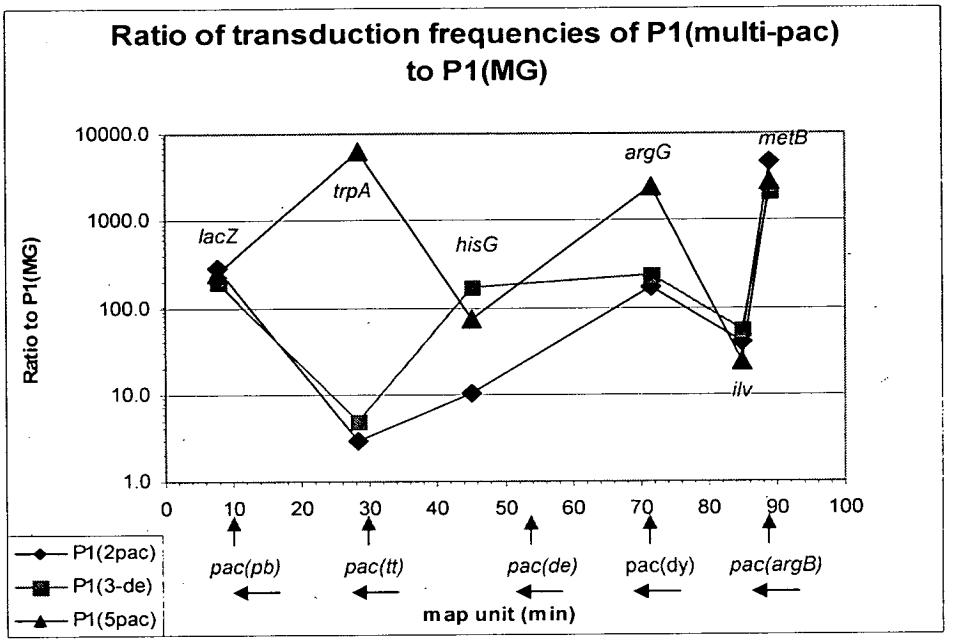


Fig 4.20 Ratio of transduction frequencies of in various P1 (multi-*pac*) lysates to that of P1 (MG) lysate. P1(2pac) has *pac*(*argB*) and *pac*(pb); P1(3de) has *pac*(*argB*), *pac*(pb) and *pac*(de); P1(5pac) contains all 5 *pacs*. Note: *pac(dy)* is located at 71.75 min while argG marker is at 71.5min. Vertical arrows are locations of corresponding *pac* sites.

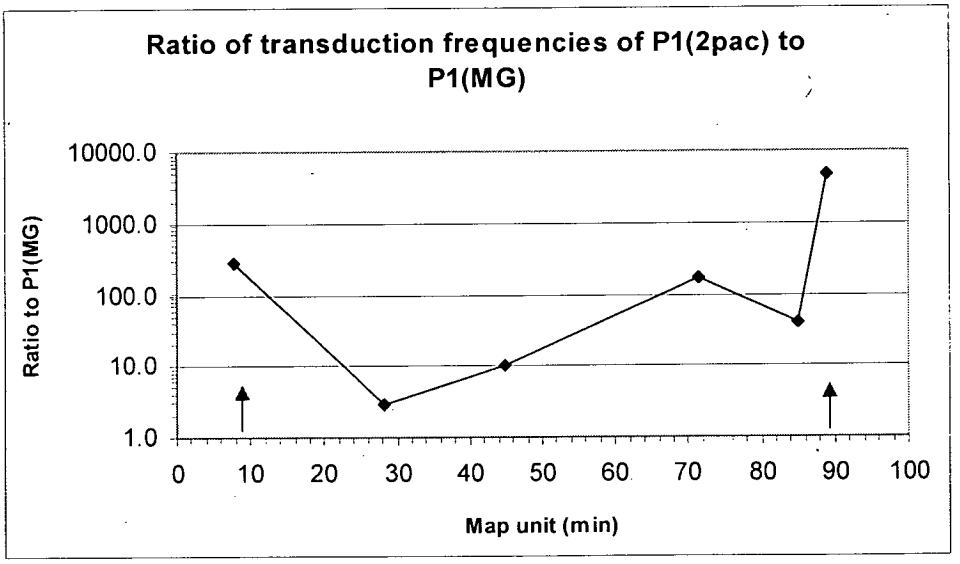


Fig 4.20.1 Ratio of transduction frequencies of P1 (2pac) lysate to that of P1 (MG).

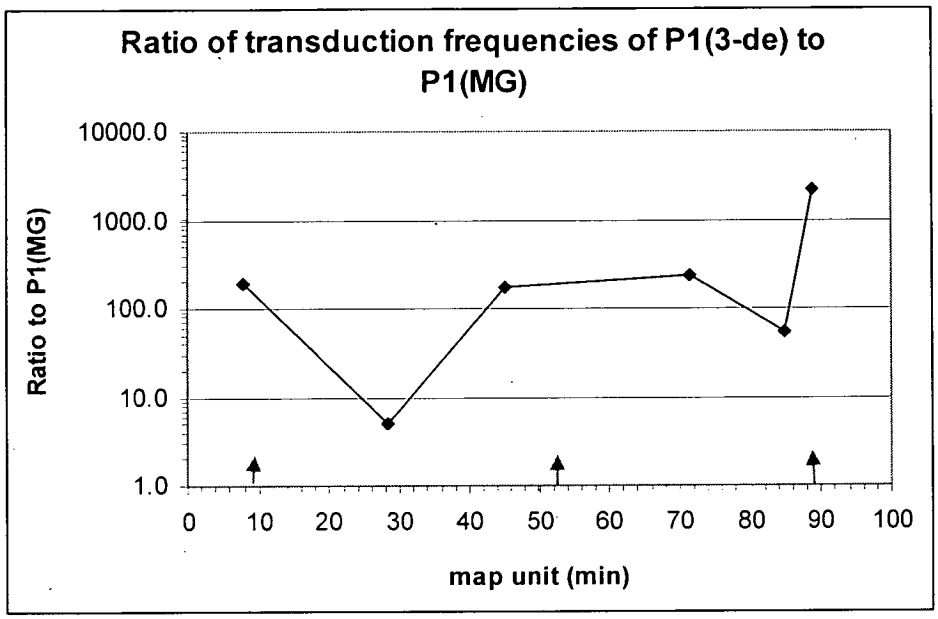


Fig 4.20.2 Ratio of transduction frequencies of P1 (3-de) lysate to that of P1 (MG).

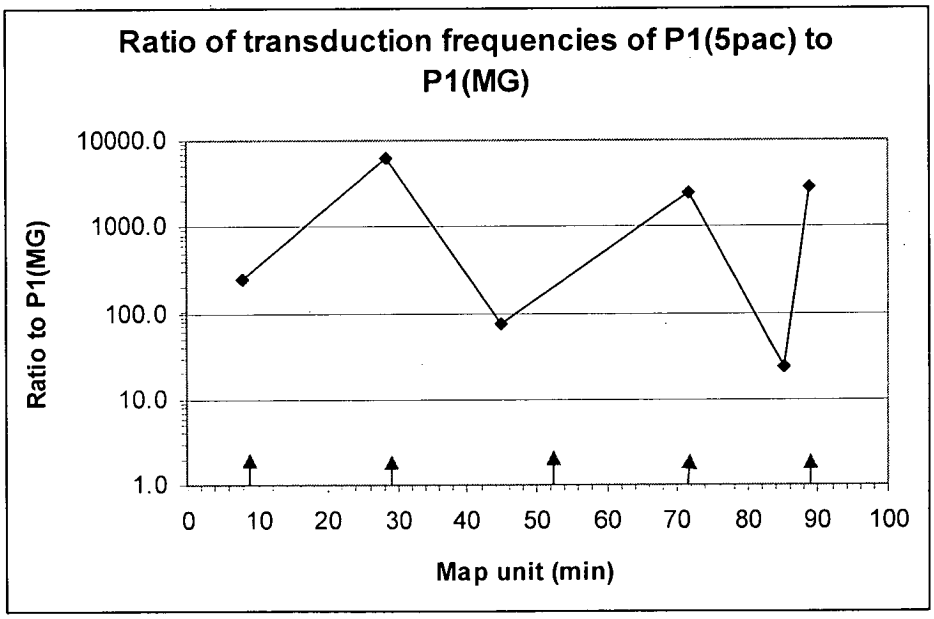


Fig 4.20.3 Ratio of transduction frequencies of P1 (5pac) lysate to that of P1 (MG).

4.3.3. Titration of P1 lysates made from multi-*pac*-containing strains

P1 plate lysates were made from different *pac*-containing strains as described in Methods and Materials. However, surprisingly, I found the titers of any strains containing two or more *pac* sites on the chromosome to be significantly lower than the titers of lysates made from wild type MG1655 or MG1655 with a single *pac*-site. The titers of MG1655 lysates could be 10^5-fold higher than lysates prepared on donors with two or more *pac* sites (Table 4.8A and B). But more interestingly, there are no big differences in the titers for any strains containing two or more *pacs*. The possible reasons will be discussed in the discussion section below.

Table 4.8 Overnight titer of whole plate titration of different strains

	Mg1655	Mg(1-arg)	Mg(1-pb)	Mg(1-de)	Mg(1-dy)	Mg(1-tt)
Fold of dilution	$x10^8$	$x10^7$	$x10^7$	$x10^7$	$x10^7$	$x10^7$
No. of plaques/0.1ml	**131**	**740**	**720**	**915**	**522**	**553**
Titer: pfu/ml	**$1.31x10^{11}$**	**$7.4x10^{10}$**	**$7.2x10^{10}$**	**$9.15x10^{10}$**	**$5.22x10^{10}$**	**$5.53x10^{10}$**
	Mg(2pac)	Mg(3-de)	Mg(3-dy)	Mg(3-tt)	Mg(4pac)	Mg(5pac)
Fold of dilution	x10	x10	x10	x10	x10	x10
No. of plaques/0.1ml	**108**	**532**	**58**	**485**	**899**	**162**
Titer: pfu/ml	**$1.08x10^4$**	**$5.32x10^4$**	**$5.8x10^4$**	**$4.85x10^4$**	**$8.99x10^4$**	**$1.62x10^4$**

A

	Mg1655	Mg(1-arg)	Mg(1-pb)	Mg(1-de)	Mg(1-dy)	Mg(1-tt)
Fold of dilution	$x10^8$	$x10^8$	$x10^8$	$x10^8$	$x10^8$	$x10^8$
No. of plaques/0.1ml	**103**	**89**	**83**	**46**	**69**	**28**
Titer: pfu/ml	**$1.03x10^{11}$**	**$8.9x10^{10}$**	**$8.3x10^{10}$**	**$4.6x10^{10}$**	**$6.9x10^{10}$**	**$2.8x10^{10}$**
	Mg(2pac)	Mg(3-de)	Mg(3-dy)	Mg(3-tt)	Mg(4pac)	Mg(5pac)
Fold of dilution	$x10^2$	x10	$x10^2$	$x10^2$	x10	$x10^2$
No. of plaques/0.1ml	**161**	**72**	**126**	**86**	**339**	**96**
Titer: pfu/ml	**$1.61x10^5$**	**$7.2x10^3$**	**$1.26x10^5$**	**$8.6x10^4$**	**$3.39x10^4$**	**$9.6x10^4$**

B

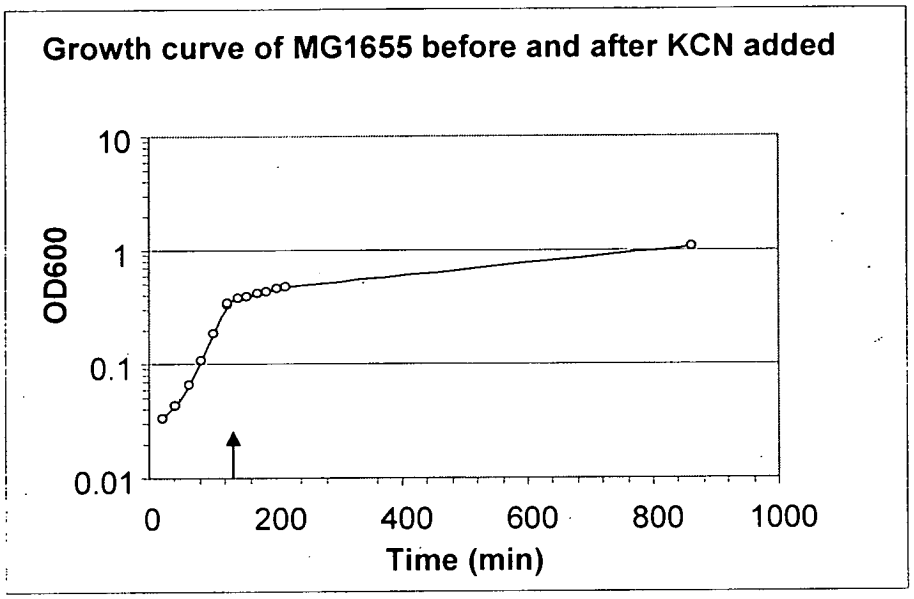
Note: Two independent experiments were done and listed in Table A and B respectively. P1 lysates were serially diluted to the desired concentration. 100 µl of diluted lysates were mixed with 100 µl of MG1655 and incubated at 37°C for 15min. 3 ml of top BBL agar with Ca^{2+} was added to each mixture and mixed evenly, and then poured onto BBL plates. Incubation was carried out at 37°C overnight. Therefore, No. of plaques per 0.1 ml in the table is the actual number on the plate.

4.3.4. Single cycle lysis curves of multi-*pac*-containing strains by P1

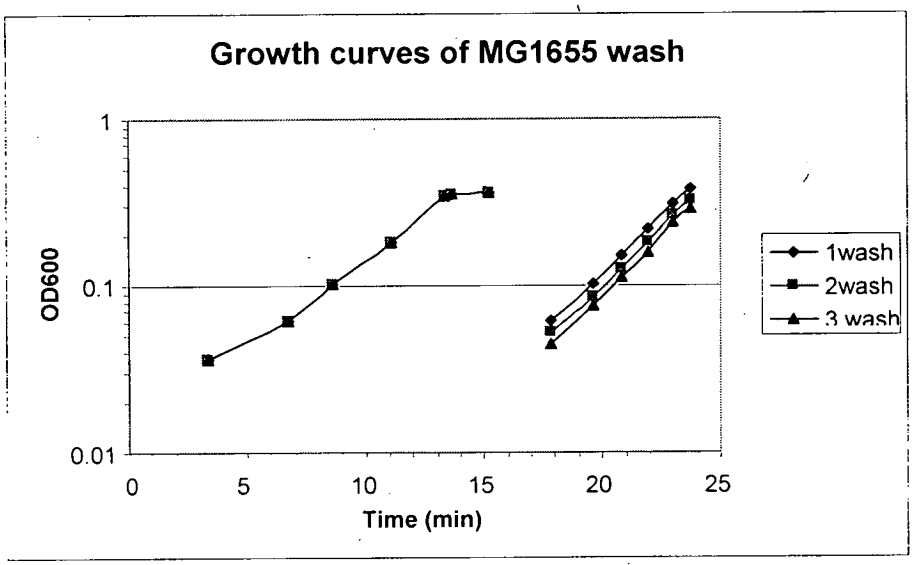
During the preparation of plate lysates, the effect of lowered yield will be amplified as the cells outgrow the phage. In order to document how phage titer was being reduced, a single cycle of infection was examined. Titration and transduction were followed in order to understand what is going on in the cell during phage development.

Cells were infected by P1 as described in Methods and Materials. CN^- was used during the absorption of P1 into cells to prevent phage development during the adsorption period (Fig 4.21 A). A centrifugation and wash step was added after P1 was adsorbed (Fig 4.21 B) to remove Ca^{2+} and CN^-. After resuspension in the same volume of fresh LB, cells were grown at 37°C and 1ml aliquots taken at the indicated times, followed by separation of supernatants and cell pellets. This is because we tried to compare released and intracellular phages, since it is worth knowing whether or not the transducing particles will be trapped in cells after infection by P1(5pac). Chloroform was then added to each sample to complete lysis. Transduction and titration were done on both supernatant and pellet samples and data are shown below (Fig 4.23; Table 4.9). An interesting phenomenon found in this work is that when strains containing more than one *pac* site were infected by P1, the time when lysis occurred was significantly delayed compared to the null and one-*pac*-containing strains (Fig 4.22). Moreover, delay in lysis was dependent on multiplicity of infection. When m.o.i. was raised, the amount of delay was reduced (Fig 4.24). This suggests that, in the multi-*pac* strain, there is competition between chromosomal and phage packaging sites for a material in limited supply. Increasing the number of genes supplying it in each cell, by increasing m.o.i results in reduced lysis delay and, very likely, a concomitant increase in viable phage titer (this has not been measured).

In the work shown in Fig 4.22, the time at which lysis begins in the 5-*pac* strain was about 20min delayed relative to that for MG1655. Titration and transduction analysis of the aliquots showed that after a single cycle of infection, the total yield of infectious P1 from MG1655 is 10-fold higher than that from the 5-*pac* containing strain (Table 4.9), while the *met* transducing frequency is hundreds-fold lower. Both infective and transducing phages were produced earlier from MG1655. Some infectious particles were possibly trapped in *pac*-containing cells, since fewer 5-*pac* containing cells were lysed than were the MG1655 wild type cells. A hypothesis to explain these observations is presented below.

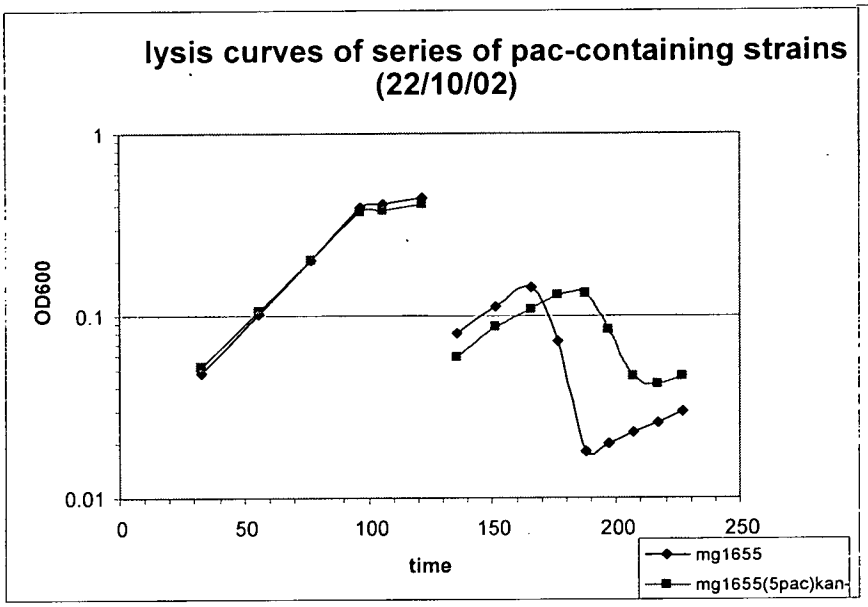


A

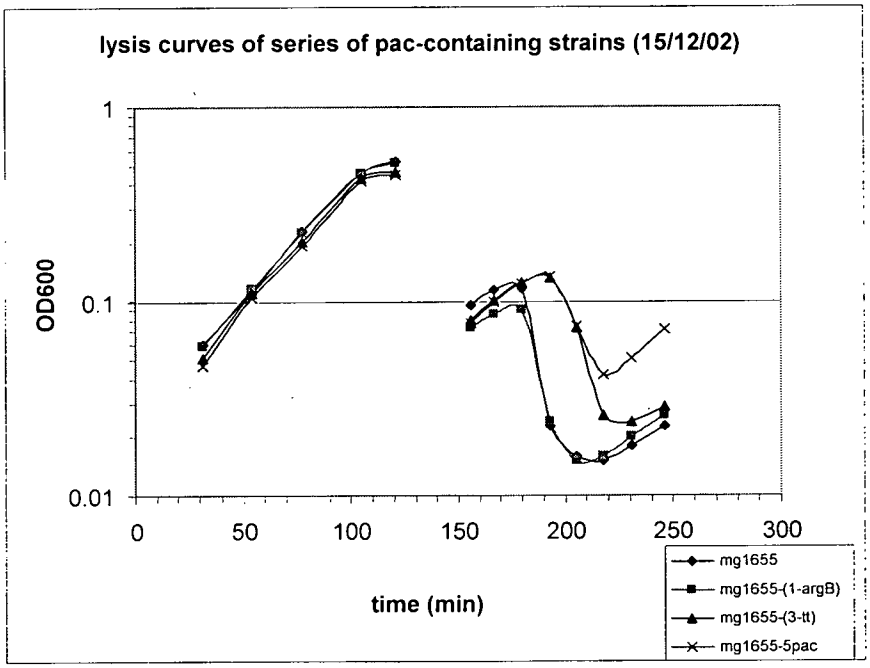


B

Fig 4.21 The effect of CN^- and washing on cell growth. **A)** CN^- was added as described in Materials and Methods and blocked the growth of MG1655 indefinitely. The arrow shows the time when CN^- was added. **B)** Washing step added as described in Materials and Methods: original growth rate restored



A



B

Fig 4.22 Lysis curves of several *pac*-containing strains after infection with P1. **A)** Diamonds are for MG1655 and squares are for MG1655(5pac). **B)** Diamonds are for MG1655, squares are for MG1655(1-arg), triangles is for MG1655(3-tt) and crosses are for MG1655(5pac).

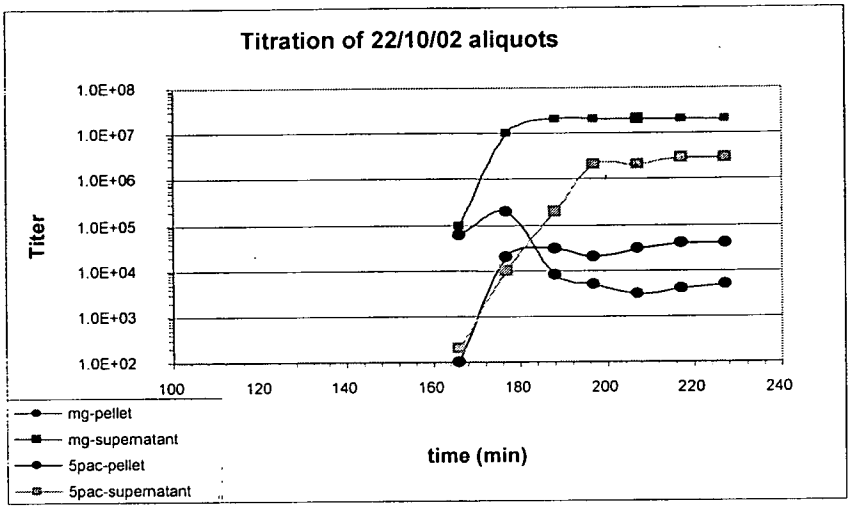


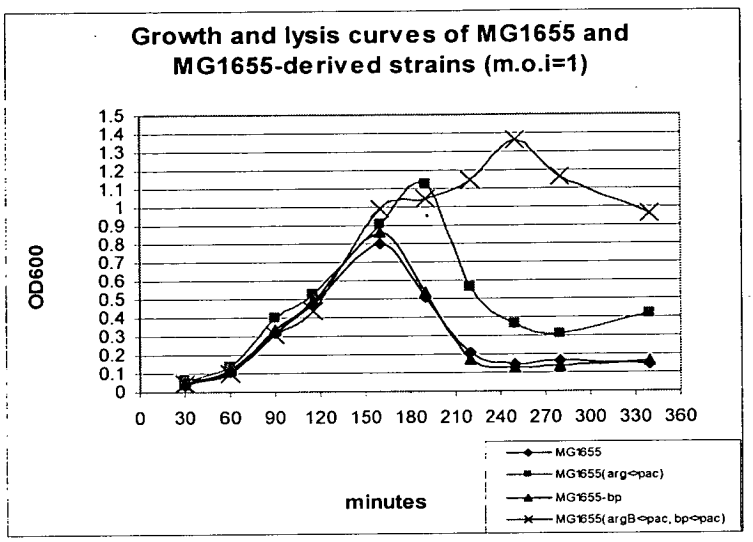
Fig 4.23 Titration of supernatants and pellets of 22/10/02 aliquots. Diamonds are for MG1655 pellet samples and are for MG1655(5pac) pellet samples. Squares are for MG1655 supernatant samples and are for MG1655(5pac) supernatant samples.

Table 4.9 Titers of pellets and supernatants, and numbers of transductants of 22/10/02 aliquots

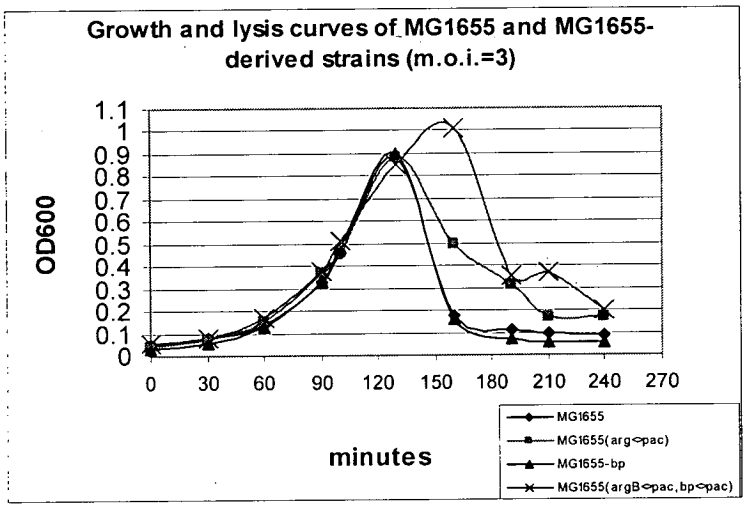
Time (min)	MG1655					MG1655(5pac)				
	OD_{600}	Titer pfu/ml		Tran. /ml	**No./ phage**	OD_{600}	Titer pfu/ml		Tran. /ml	**No./ phage**
		#Pellet	Supern	S.P.			#Pellet	Supern	S.P.	
166	0.143	6×10^6	**1×10^7**	**30**	**$3x10^{-6}$**	0.109	1×10^2	**2×10^4**	**0**	**0**
177	0.072	2×10^7	**1×10^9**	**4660**	**$5x10^{-6}$**	0.129	2×10^4	**1×10^6**	**2000**	**$2x10^{-3}$**
188	0.018	8×10^5	**2×10^9**	**8660**	**$4x10^{-6}$**	0.134	3×10^4	**2×10^7**	**20000**	**$1x10^{-3}$**
197	0.020	5×10^5	**2×10^9**	**8130**	**$4x10^{-6}$**	0.084	2×10^4	**2×10^8**	**257000**	**$1x10^{-3}$**
207	0.023	3×10^5	**2×10^9**	**9600**	**$5x10^{-6}$**	0.046	3×10^4	**2×10^8**	**356000**	**$2x10^{-3}$**
217	0.026	4×10^5	**2×10^9**	**9150**	**$5x10^{-6}$**	0.042	4×10^4	**3×10^8**	**460000**	**$2x10^{-3}$**
227	0.030	5×10^5	**2×10^9**	**8370**	**$4x10^{-6}$**	0.046	4×10^4	**3×10^8**	**453000**	**$2x10^{-3}$**

 # Pellet: after separation of pellets and supernatants, Pellets were resuspended in 0.5ml phage buffer and chloroform was added to each aliquot. Titration steps are described below. Therefore, total phage particles in cells are equal to ½ of the number presented in the column above.

Note: MM303 was grown to OD600 = 1-1.5. 100µl aliquot of each P1 lysate was mixed with 100µl of MM303, while 100µl of each 100-fold diluted P1 (5pac) aliquot was mixed with 100µl of MM303. The mixtures were then incubated at 37oC for 15min. 2.5ml of top water agar was added to the mixture and poured onto the *mef* plates. 1ml aliquots were taken at the indicated times. Supernatants and cell pellets were separated by centrifugation. Pellets were then resuspended in 0.5ml phage buffer and lysis was completed by adding chloroform. Spot titration was carried out as described in Methods and Materials.



A



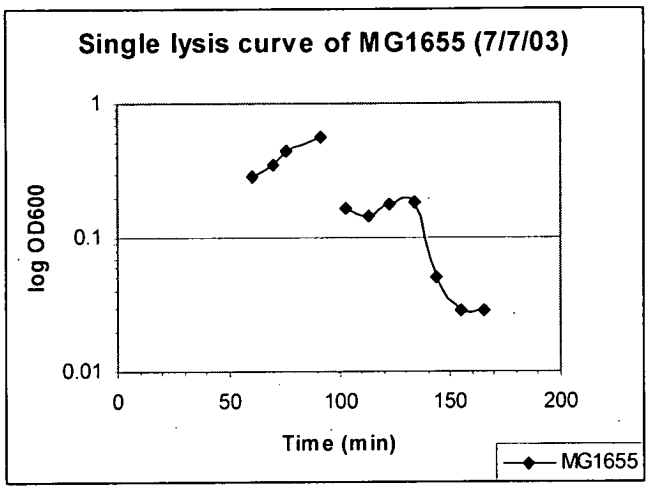
B

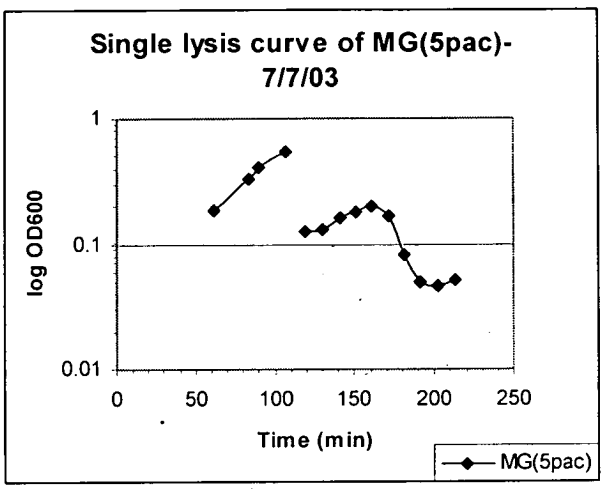
Fig 4.24 lysis curves of MG1655 and pac-containing strains with different m.o.i. Lysis was carried out as described in M. and M. with the indicated m.o.i. No CN^- and wash step was used in this work. diamonds are MG1655; the square is MG1655 (1-arg); the triangle is MG1655 (1-pb) and the cross is MG1655 (2pac). **A)** P1 was added with a m.o.i of 1; **B)** P1 was added with a m.o.i of 3. With the increase of m.o.i. from 1 to 3, the delay of lysis time got shorter. Note: Since the m.o.i. in this work is lower than that in Fig 4.21 and no wash steps were added, therefore cannot prevent multiple infections, the curve pattern of single pac strain appears different.

4.3.5. P1 DNA concentration in P1-infected cells

In the previous section I showed that the phage yield from strains with multiple chromosomal *pac* sites appears to be reduced and that lysis is delayed. Increasing the multiplicity of infection partly overcame the problem, helping lysis to occur earlier. However, we have no information regarding what is happening inside the cells to cause these effects. A possibility is that phage DNA synthesis is prevented or delayed because the chromosomal *pac* sites are competing for a protein required for replication. In this section I will describe experiments designed to follow phage DNA synthesis after infection. Lysates were made on MG1655 and on MG-5 *pac* using a KCN washing procedure to allow a single cycle of synchronous infection. Cells grown in broth to OD_{600} of 0.4 – 0.5 were infected at an m.o.i. of 4, as used in the experiment reported in Fig 4.22. After washing and resuspension, aliquots were taken at the indicated times and labelled MG 0'(aliquot taken right after resuspension), MG 10', MG 20', MG 30', MG 40' and 5 *pac* 0', 5 *pac* 10', 5 *pac* 20', 5 *pac* 30', 5 *pac* 40', 5 *pac* 50' respectively (Fig 4.25; Table 4.10). Total cellular DNA was extracted as described in Materials and Methods and Southern blots used to measure the P1 DNA concentration in the samples (Fig 4.26; Fig 4.27). Equal amounts of each sample, about 0.075μg/well, were loaded onto the agarose gel after digestion by *Eco*RI. A probe for P1 DNA was prepared as follows: pSHI93-Δ4, a pBR322-based plasmid containing the truncated P1 *darA* operon, was labelled by random priming. A probe for the chromosomal marker, yeeA, was also used in an attempt to measure the amount of total chromosomal DNA in each sample.

As we can see on the filter (Fig 4.27), after overnight hybridisation, two bands, 16.5k (P1 *Eco*RI 1 fragment) and 2.8K (P1 *Eco*RI 12 fragment) respectively, appeared. An extra band visible in lanes of 5 *pac* 50' DNA (obvious) and 5 *pac* 40' DNA (weak), of about 4.4kb, is probably the *yeeA* band. The absence of signals for chromosomal DNA in other lanes may result from there being too little chromosomal DNA available in these lanes.





A B

Fig 4.25 Lysis of P1-infected MG1655 and MG1655(5 *pac*). CN^- was added to stop cell growth during absorption at 76min for MG1655 experiment and at 90min for MG(5 *pac*) experiment. The discontinuity in O.D. represents a dilution.

Table 4.10 OD_{600} values and titers of the aliquots taken at the indicated times

time	OD_{600}-MG	Titer pfu/ml		time	OD_{600}-5pac	Titer pfu/ml
61	0.284			61	0.185	
70	0.355			83	0.336	
(+) 76	0.45	P1+ CN+		(+) 90	0.409	P1+ CN+
92	0.562			107	0.538	
	5 x dilu.	*Wash+resusp.*			*5 x dilu.*	*Wash+resusp.*
(MG 0') 103	0.168	1×10^3		(5 *pac* 0') 11	0.127	5×10^3
(MG 10') 113	0.146			(5 *pac* 10') 13	0.132	1×10^4
(MG 20') 123	0.178	5×10^6		(5 *pac* 20') 141	0.162	1×10^4
(MG 30') 134	0.183	2×10^8		(5 *pac* .30') 151	0.181	2×10^4
(MG 40') 144	0.05	5×10^8		(5 *pac* .40') 161	0.202	8×10^6
155	0.029	3×10^8		(5 *pac* .50') 171	0.17	1×10^8
165	0.029	5×10^8		181	0.082	3×10^8
				191	0.049	3×10^8
				202	0.047	3×10^8
				213	0.052	

Note: ODs were plotted against time in Fig 4.24. **(+):** time of addition of P1 and CN^-. P1 absorption was carried out for about 20min and it took about 12min for washing and resuspending cells to remove Ca^{2+} and CN^-.

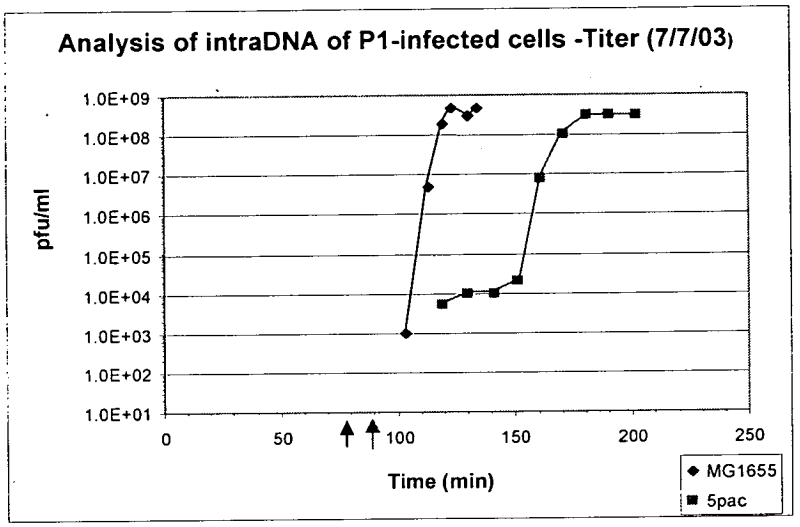


Fig 4.26 Titer of the supernatant of P1-infected MG1655 and 5 *pac* strains following the time course. Arrows show the time of infection before washing step.

In Fig 4.25 and Table 4.10 we see that rapid lysis of the MG1655 culture begins after about 35 min of phage development, while lysis of the 5- *pac* strain is delayed until about 45 minutes. The eventual titers of infectious phage reach about the same level for both cultures. This observation is somewhat inconsistent with the previous observations (see Fig 4.23 and Table 4.9) where 10-fold differences were observed. One possibility is that the efficiency of stopping by the CN^- used in this experiment was not as good as it was in the previous work, since the KCN solution was probably too old.

The titer of the supernatant of P1-infected MG1655 increased 1000-fold by 20min after re-growing and reached the maximum level 30min after re-growing, while the titer of the supernatant of P1-infected MG1655 (5 *pac*) increased only slowly and maintaining a level of at 10^4 for at least 20min, and then dramatically increased to 10^6 10min later and reached the maximum level 50min after re-growing (Fig 4.26). Then, we proceeded to measure the phage DNA content of the samples.

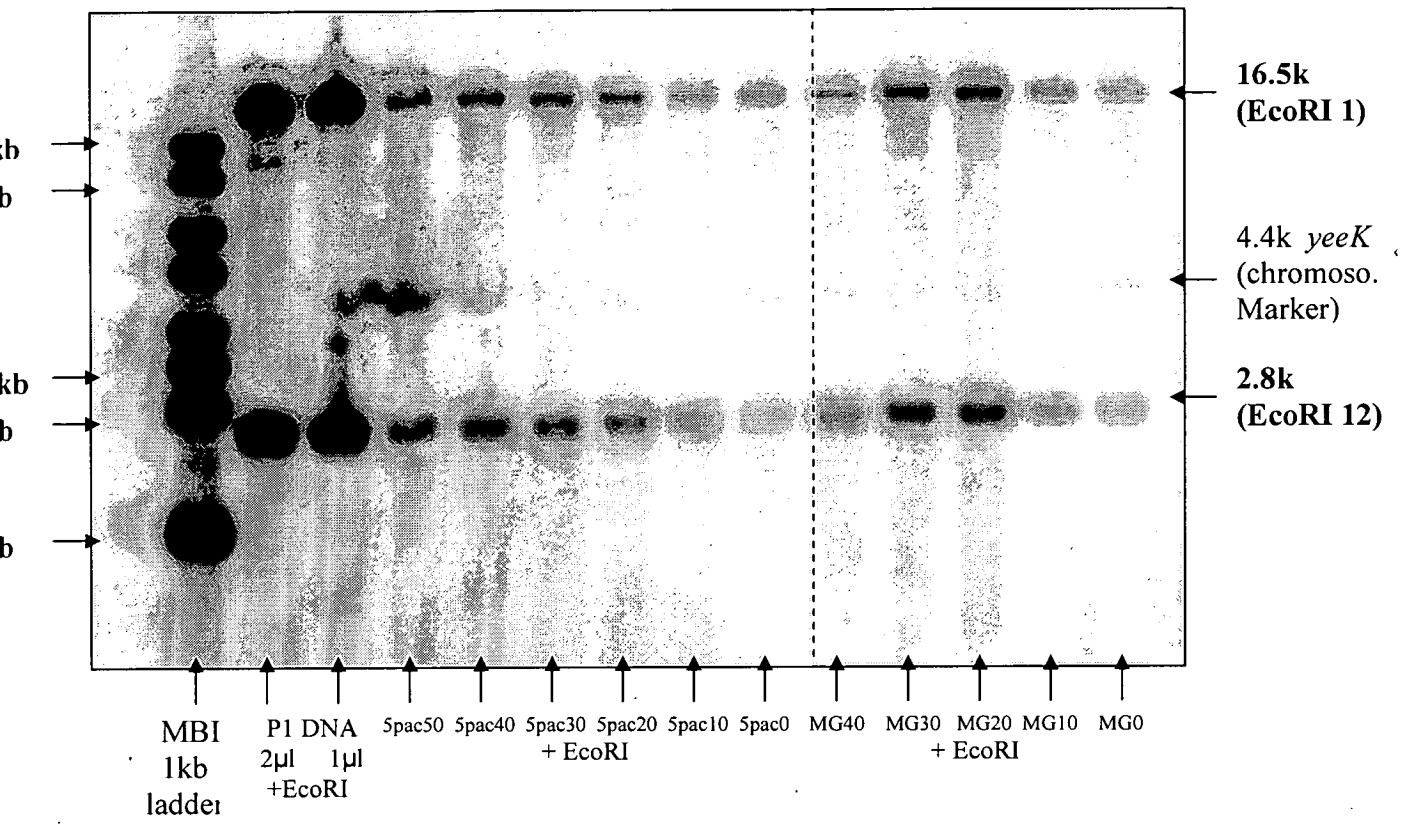


Fig 4.27 Southern blot of P1 DNA purified from P1 infected cells (**2/7/03**). Details have been described in the text.

Using Imagequant, the area of each band is obtained. The background of each lane has been removed separately by subtracting the background area of that lane from the areas of the bands within it. Since the blot signals represent the total DNA in the cells that were pelleted, to compare the intracellular P1 DNA of MG1655 with that of MG1655(5 *pac*) at various times, the same number of each type of cells should be compared at each time. Therefore, all area values are adjusted for OD, in terms of area value / OD600, given that OD value is proportional to the number of cells (Table 4.11, 4.12; Fig 4.29, 4.30).

Table 4.11 Analysis of the densities of the bands from Southern blot using Imagequant

PEAK	AREA	bg AREA	corrected AREA	OD_{600}	Relative P1DNA/cell
5 *pac* 50'	131125	12471	118654	0.17	**698**
5 *pac* 40'	140856	13229	127627	0.202	**632**
5 *pac* 30'	97884	13289	84595	0.181	**467**
5 *pac* 20'	66080	8809	57271	0.162	**354**
5 *pac* 10'	25409	4217	21192	0.132	**161**
5 *pac* 0'	14394	2969	11425	0.127	**90**
MG40'	46486	3330	43156	0.05	**863**
MG30'	136013	5696	130317	0.183	**712**
MG20'	149482	6861	142621	0.178	**801**
MG10'	27290	956.9	26333.1	0.146	**180**
MG0'	18471	793.6	17677.4	0.168	**105**

A

PEAK	AREA	bg AREA	corrected AREA	OD_{600}	Relative P1DNA/cell
5 *pac* 50'	132434	9276	123158	0.17	**724**
5 *pac* 40'	139124	8310	130814	0.202	**648**
5 *pac* 30'	95074	6031	89043	0.181	**492**
5 *pac* 20'	65870	4094	61776	0.162	**381**
5 *pac* 10'	23980	2237	21743	0.132	**164**
5 *pac* 0'	15818	2210	13608	0.127	**107**
MG40'	40995	2858	38137	0.05	**763**
MG30'	122324	6409	115915	0.183	**633**
MG20'	146003	7794	138209	0.178	**776**
MG10'	25152	1644	23508	0.146	**161**
MG0'	19055	1803	17252	0.168	**103**

B

Note: the measured band density has been corrected by subtracting the background density. The OD_{600} value of each sample was used to normalize the densities in order that P1 DNA from equal numbers of cells could be compared. As expected, 2 bands for each sample were obtained, one is 2.8kb and the other is 16.5kb (see Fig 4.25). Therefore, both groups of bands were analyzed using Imagequant. "bg" means "background". Table A) the results for the bands whose sizes are 2.8kb. Table B) the results for the bands whose sizes are 16.5kb.

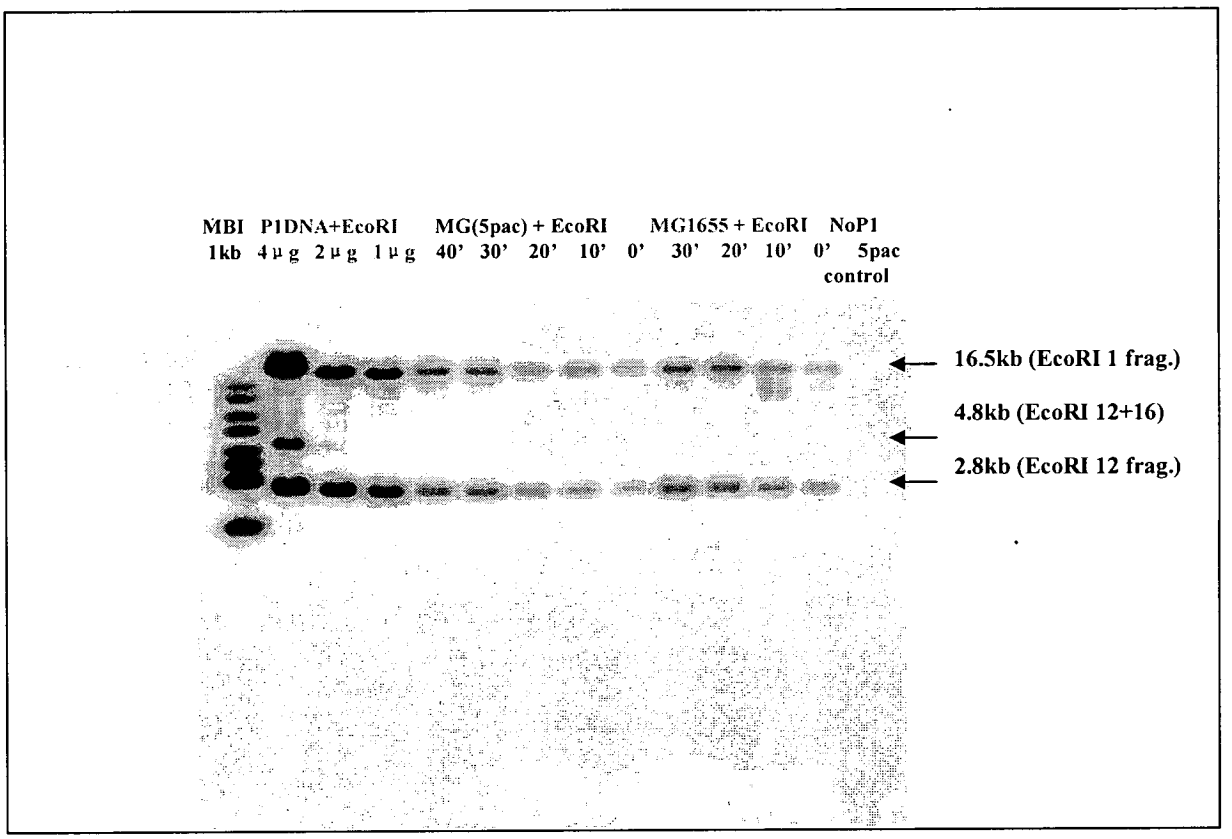


Fig 4.28 Detection of P1 DNA concentration in P1-infected cells by Southern blot (9/5/03). The experiment was carried out as described for Southern blot in Fig 4.25. Data were collected using Imagequant as described above. Two bands as mentioned above were obtained. The extra band appearing in 4 µg P1 DNA was probably due to the partial digestion of fragment EcoRI 12 and EcoRI 16, which results in a 4.8kb band.

Table 4.12 Analysis of the densities of the bands from Southern blot using Imagequant

PEAK	AREA	bg AREA	corrected AREA	OD	Relative P1DNA/cell
5 *pac* 40'	260127	7841	252286	0.874	**289**
5 *pac* 30'	215881	6743	209138	0.803	**260**
5 *pac* 20'	86973	2975	83998	0.731	**115**
5 *pac* 10'	107255	1752	105503	0.634	**166**
5 *pac* 0'	48318	1499	46819	0.478	**98**
MG30'	229311	3589	225722	0.534	**423**
MG20'	230914	4213	226701	0.702	**323**
MG10'	106643	1413	105230	0.624	**169**
MG0'	43699	435.1	43264	0.51	**85**

A

PEAK	AREA	bg AREA	corrected AREA	OD	Relative P1DNA/cell
5 *pac* 40'	253916	14518	239398	0.874	**274**
5 *pac* 30'	231403	18697	212706	0.803	**265**
5 *pac* 20'	81961	5376	76585	0.731	**105**
5 *pac* 10'	92230	5278	86952	0.634	**137**
5 *pac* 0'	38530	1859	36671	0.478	**78**
MG30'	236737	12771	223966	0.534	**419**
MG20'	218960	11738	207222	0.702	**295**
MG10'	136190	9531	126659	0.624	**203**
MG0'	49851	2873	46978	0.51	**92**

B

Note: the measured band density was corrected by subtracting the background density. The OD600 value of each sample was used to normalize the densities in order that P1 DNA from equal numbers of cells could be compared. "bg" means "background". Table A) analysis of the 2.8 kb bands. Table B) analysis of the 16.5 kb bands.

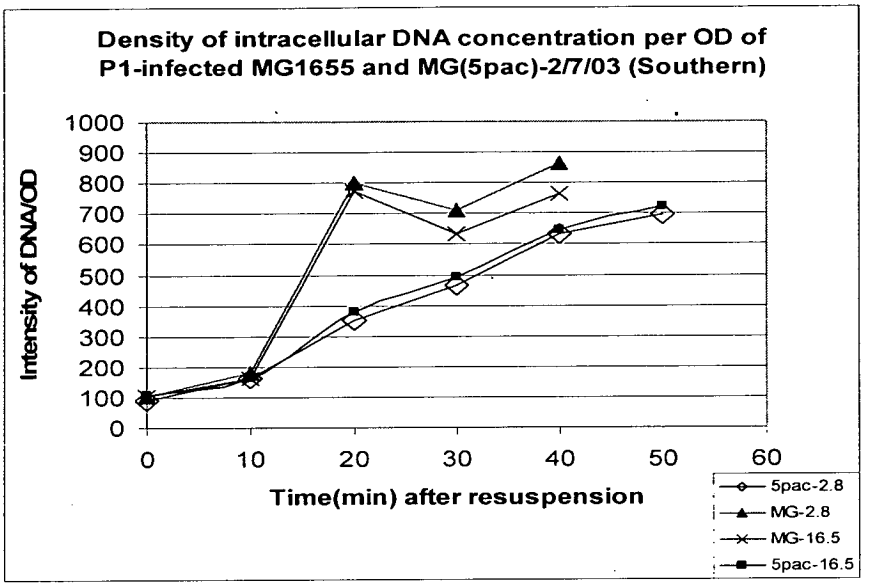


Fig 4.29 Densities of the bands from Southern blot in Fig 4.25. The density of each band has been normalized to OD_{600} in order that comparison was carried out among the equal number of cells. Time zero "0" in the diagram is the time when P1-absorbed cells were resuspended in the fresh LB after absorption for 15min in the presence of CN, washing and resuspension.

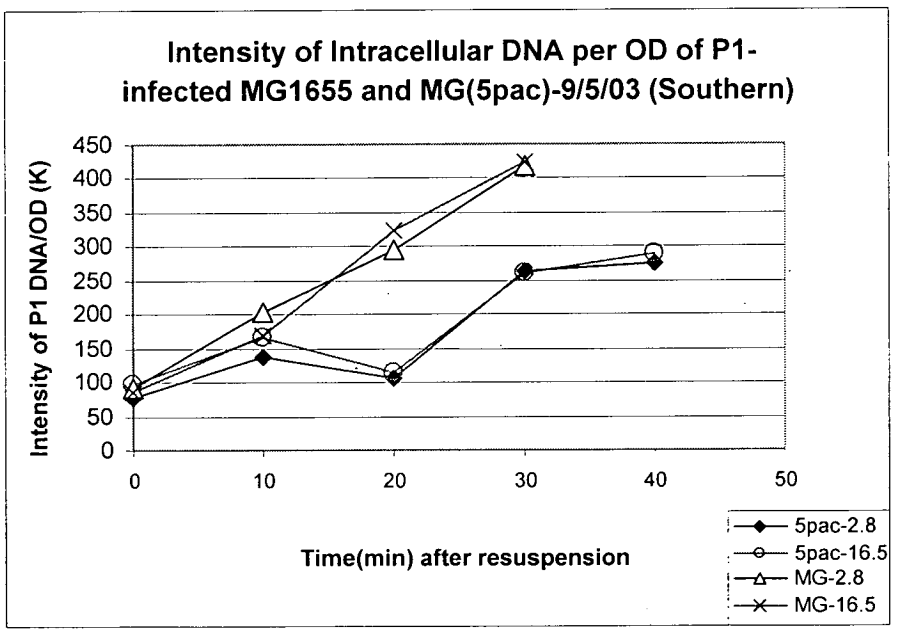


Fig 4.30 Densities of the bands from Southern blot in Fig 4.26. The density of each band has been normalized to OD_{600} in order that comparison was carried out among the equal number of cells. Time zero "0" in the diagram is the time when P1-absorbed cells were resuspended in the fresh LB after absorption for 15min in the presence of CN, washing and resuspension.

From the above data, we can clearly see that, just after infected cell resuspension, similar amounts of P1 DNA were detected in each strain. However, the P1 DNA concentration in the MG1655 rapidly increases and by 20min, had nearly reached its maximum concentration which was then maintained until cell lysis. In contrast, the P1 DNA concentration in MG1655 (5 *pac*) increased gradually from the beginning and was still continuing to rise as the last sample was taken at 50 min. In addition to the above observation that production of P1 DNA in MG1655 (5 *pac*) is slowed relative to MG1655, the total amount of P1 DNA produced in MG1655 is also higher than that of P1 DNA produced in MG1655 (5 *pac*).

In this section, we have looked into the production of P1 DNA in the P1-infected cells. The P1 DNA was synthesized slower in P1 infected MG(5pac) cells than that in P1 infected MG1655 cells. Also, the total amount of P1 DNA produced in MG(5pac) is lower than that of P1 DNA produced in MG1655. This is consistent with the observation in previous titration experiments (Fig 4.23 and Table 4.9). Although more work is required to further explain this observation, it is possible that phage DNA synthesis is prevented or delayed in multiple-*pac* containing strains because the chromosomal *pac* sites are competing for a protein required for replication.

4.3.6 P1 packaging of chromosomal DNA from multi-*pac* strains: southern blotting experiments

Table 4.7 and Fig 4.20 show that transduction of markers to one side of *pac is* greatly stimulated, even when there are several *pac sites* in a single chromosome. In order to directly determine whether packaging of such markers is stimulated to the same degree as are their transductions, Southern blots were performed to examine the packaging of the chromosomal markers of multi-*pac* strains. P1 lysates were made from each strain and P1 DNA was extracted as described in Materials and Methods. 10 *pac*-containing strains in total were examined and MG1655 was used as a control (Table 4.13). Southern blots were carried out and probes were prepared as described above.

Table 4.13 List of names of *pac*-containing strains which were examined by Southern blotting.

Strain's names	No. of *pac* sites	Position of *pac* sites (min)	P1 lysates' names
MG1655(1-arg)	**1**	**90**	**P1 (1-arg)**
MG1655(1-pb)	**1**	**9**	**P1 (1-pb)**
MG1655(1-de)	**1**	**54**	**P1 (1-de)**
MG1655(1-dy)	**1**	**75**	**P1 (1-dy)**
MG1655(1-tt)	**1**	**29**	**P1 (1-tt)**
MG1655(2pac)	**2**	**90; 9**	**P1 (2pac)**
MG1655(3-de)	**3**	**90; 9; 54**	**P1 (3-de)**
MG1655(3-dy)	**3**	**90; 9; 75**	**P1 (3-dy)**
MG1655(3-tt)	**3**	**90; 9; 29**	**P1 (3-tt)**
MG1655(4pac)	**4**	**90; 9; 75; 29**	**P1 (4pac)**
MG1655(5pac)	**5**	**90; 9; 75; 54; 29**	**P1 (5pac)**
MG1655	**0**		**P1 (MG)**

Data were analyzed using Imagequant and shown in Figs 4.33 and 4.34. In Fig 4.33, we tested 10 markers. As we can see, the packaging of *atpG*, *metB*, *yihU* and *hemD* (locations as shown in the Fig)

were increased by *pac(*arg), located at 89 min, while the packaging of *dacB* (see Fig 4.31 for an explanation of *dacB* band size) was increased by *pac* (dy), located at 72 min. *trp*, located at 28 min was used to test *pac* (tt) and the increase of its packaging was observed as expected. The size of *dacB* marker looks smaller in strains containing *pac* inserted between *dacB* and *yhbZ* than in strains without *pac* at that location. This is due to *pac* cleavage within the larger fragment (see Fig 4.31). It is not surprising that *yjfJ*, *holC* and *ybaV* packaging was not increased by the *pac* sites since they are behind these *pac* sites and may be degraded after cutting at these sites. No indicators for *pac(*pb) and *pac(*de) were included in this filter, although it is possible that *trpA* packaging is increased in the (de) lysate. In Fig 4.34, a different set of markers including several shown in Fig 4.34 as well as new ones were tested. *yfaX*, located at 51 min for *pac*(de) and *yaeE*, located at 4.8 min for *pac*(pb) were included. The packaging of *pac*(de) is increased significantly for MG(1-de) and MG(3-de) but only slightly if at all in MG(4pac) and MG(5pac). *yaeE* in contrast shows increased packaging in all strains where it would be expected. Nonetheless, we thought it is worthwhile to verify that *pac*(de) and *pac*(pb) were functional in the 4pac and 5pac strains by testing two new probes to the markers, *phoA*, (See Fig 4.35). at 8.7 min and *intS*, (see Fig 4.37) at 53.2 min were made. The further Southern blottings showed that the *pac sites* are functional. It is also necessary to point out that more DNA was probably needed in the blots in Fig 4.33 and Fig 4.34, especially in Fig 4.34. Also, perhaps some probes were not labelled well as can be seen from chromosomal DNA. This did not seriously affect our overall results as the packaging ratio of each marker amongst strains is the data sought. However, it did cause difficulty in analyzing bands for some markers.

From these filters, we can see that all the markers close to *pac* have been packaged at high frequency and those markers immediately behind the *pac sites* have not been packaged at high frequency. In addition, a pattern of packaging peaks and troughs around *pac sites* is apparent.

An extra band appearing on the 1-dy lane and right below *trpAC* band may be due to incomplete cleavage at *pac*, in which case, a 5.8kb fragment including the entire *dacB* gene, the intact P1 EcoRI-20 fragment and a FRT-KAN cassette is generated, since strain MG (1-dy) contains the FRT-KAN cassette, which is about 1.4kb in size.

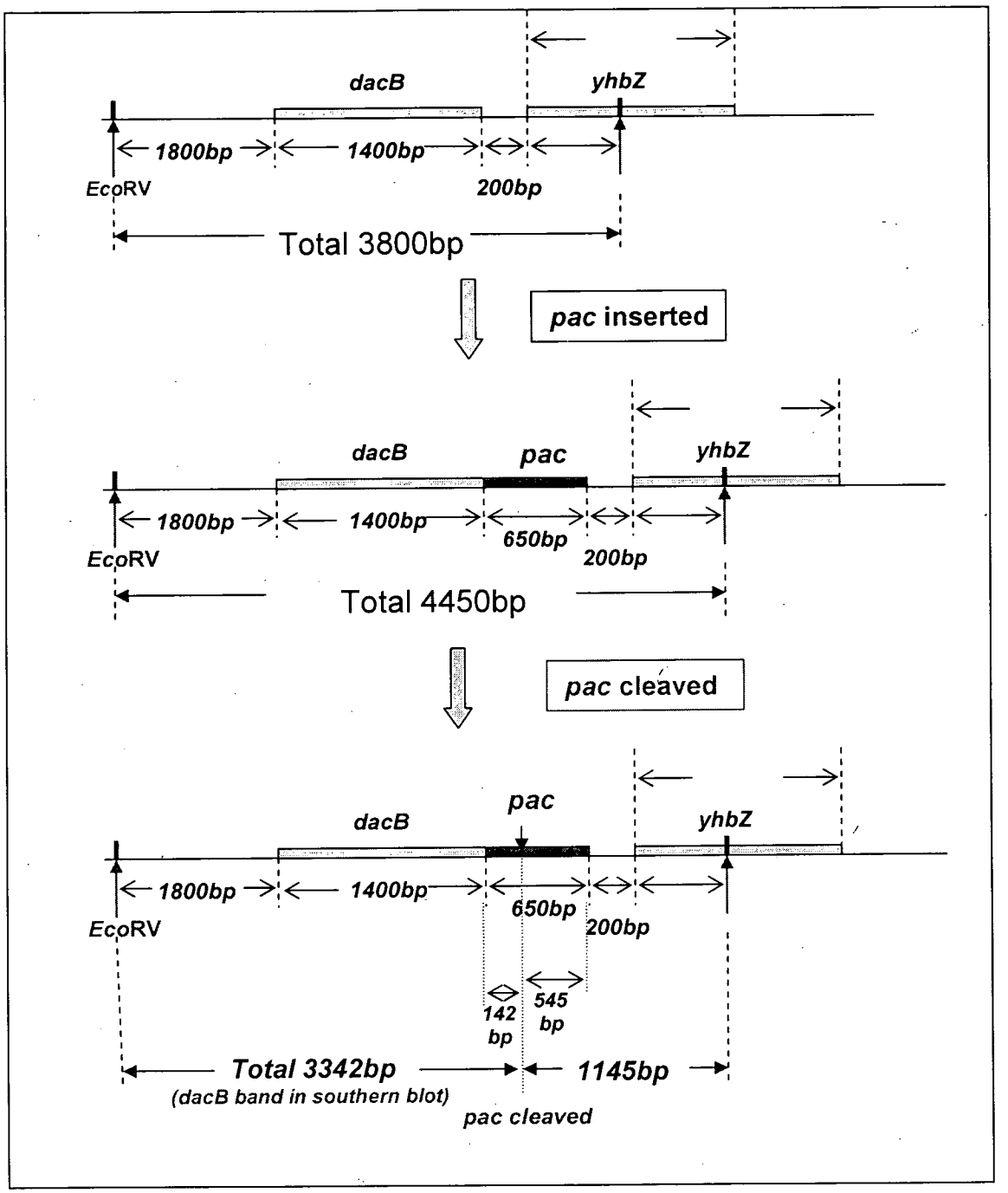


Fig 4.31 Generation of the *dacB* band digested by *Eco*RI, which appears on the southern filters.

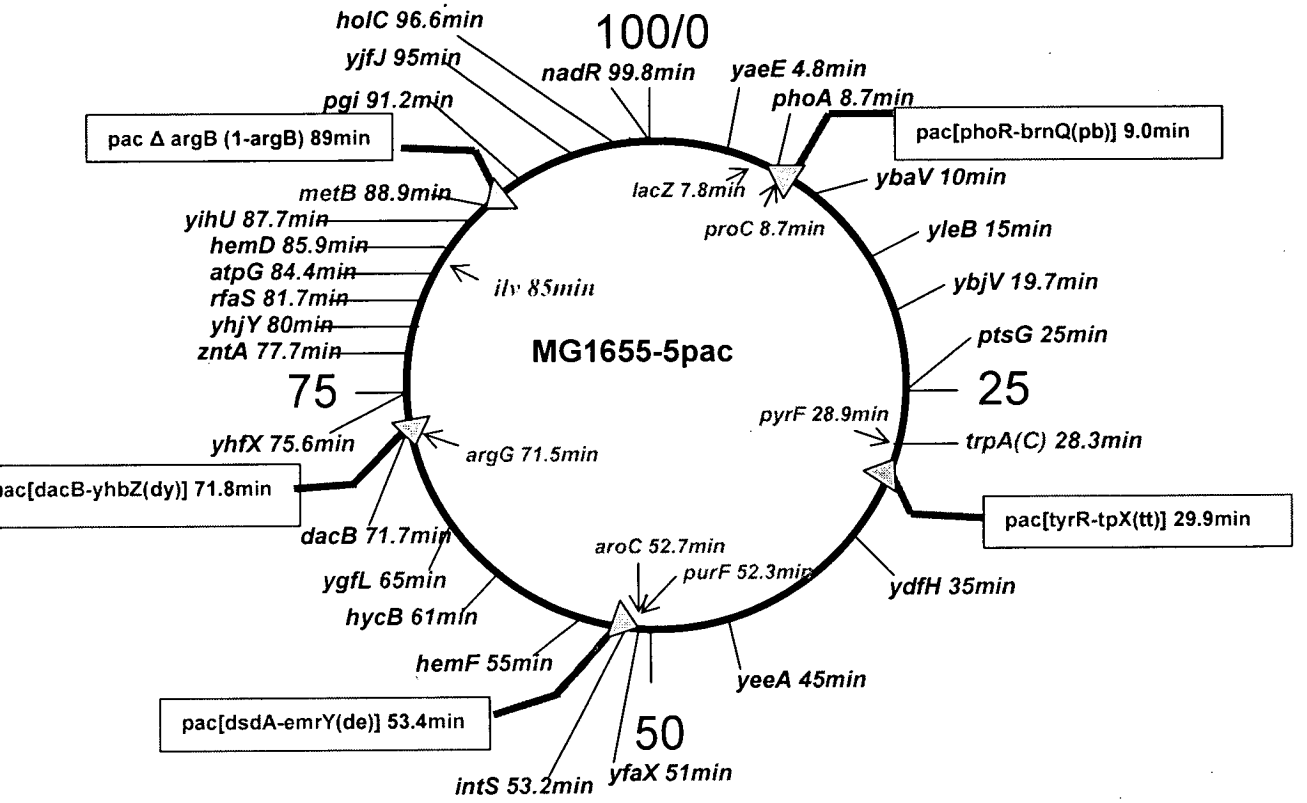


Fig 4.32 The locations of 5 *pacs* on the chromosome and markers along the entire chromosome for the probes of southern blot. The triangles stand for *pac* sites on the chromosome and are pointing as packaging will proceed. The genes outside the ring followed by their position (min) on the chromosome are for southern probes. The markers inside the ring were used for transduction.

Table 4.14 Markers for preparing the probes of Southern blot.

Marker names	location on chromosome	size by EcoRV	orientation of pac
nadR	99.8min	3.0k	
holC	96.6min	0.7k	
yjfJ	95min	1.2k	
pgi	91.2min	4.6k	
pac Δ argB-(1-arg)	**89min**		↓
metB	88.9min	8.0k	
yihU	87.7min	2.4k	
hemD	85.9min	2.0k	
atpG	84.4min	13.5k	
rfaS	81.7min	1.4k	
yhjY	80min	1.7k	
zntA	77.7min	10.8k	
yhfX	75.6min	3.0k	
pac(dacB-yhbZ)-dy	**71.75min**		↓
dacB	71.7min	3.8k	
ygfL	65min	2.5k	
hycB	61min	3.1k	
hemF	55min	13.5k	
pac(dsdA-emrY)-de	**53.4min**		↓
intS	53.2min	4.7k	
yfaX	51min	1.4k	
yeeA	45min	1.8k	
ydfH	35min	2.2k	
pac(tyrR-tpX)-tt	**29.9min**		↓
trpA(*trpC*)	28.3min	5.9k	
ptsG	25min	6.7k	
ybjV	19.7min	2.3k	
yleB	15min	4.6k	
ybaV	10min	1.8k	
pac(phoR-brnQ)-pb	**9.0min**		↓
phoA	8.7min	9.7k	
yaeE	4.8min	1.2k	

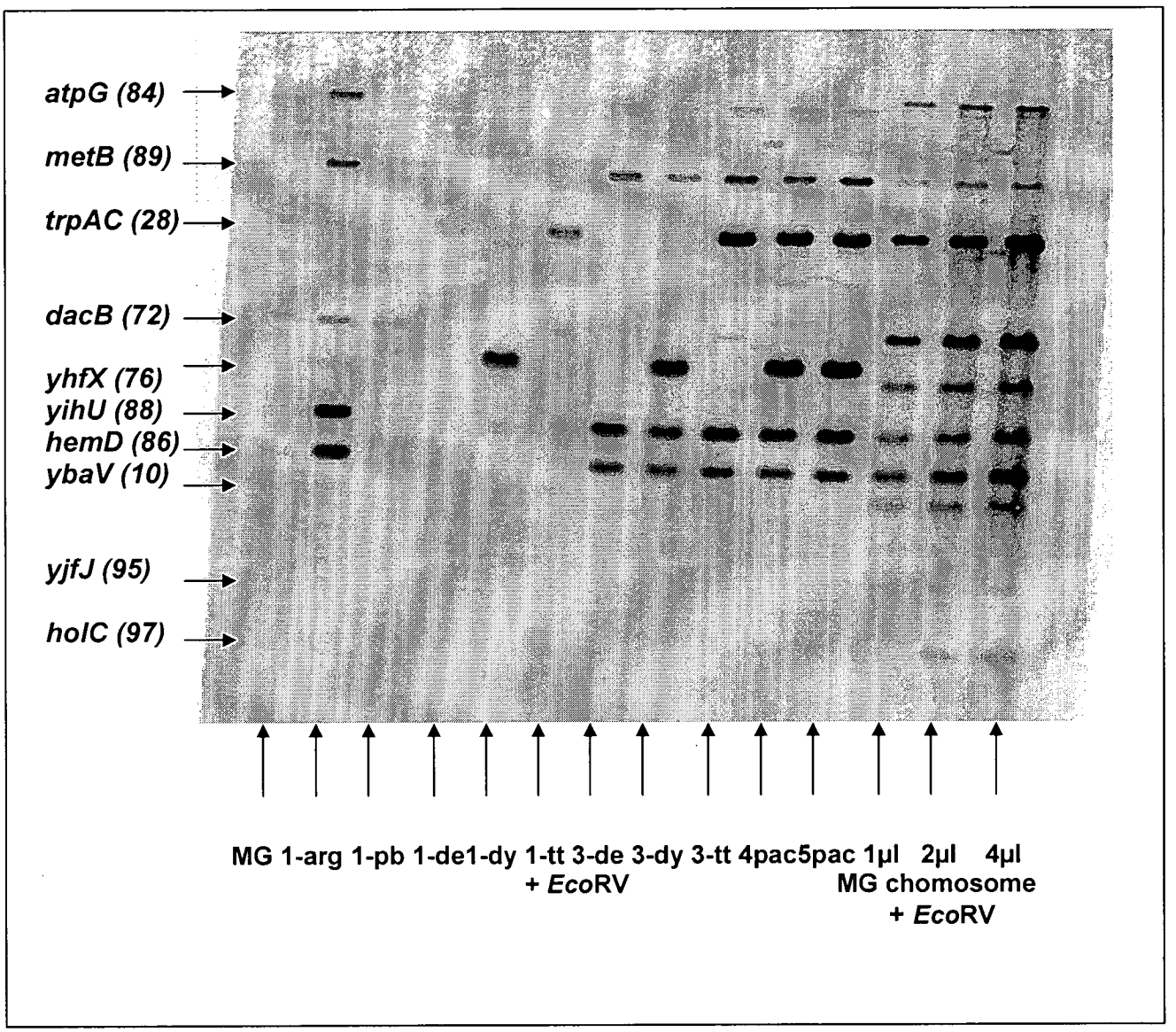


Fig 4.33 Analysis of the chromosomal packaging of multi-*pac* strains using Southern blot (17/01/03).

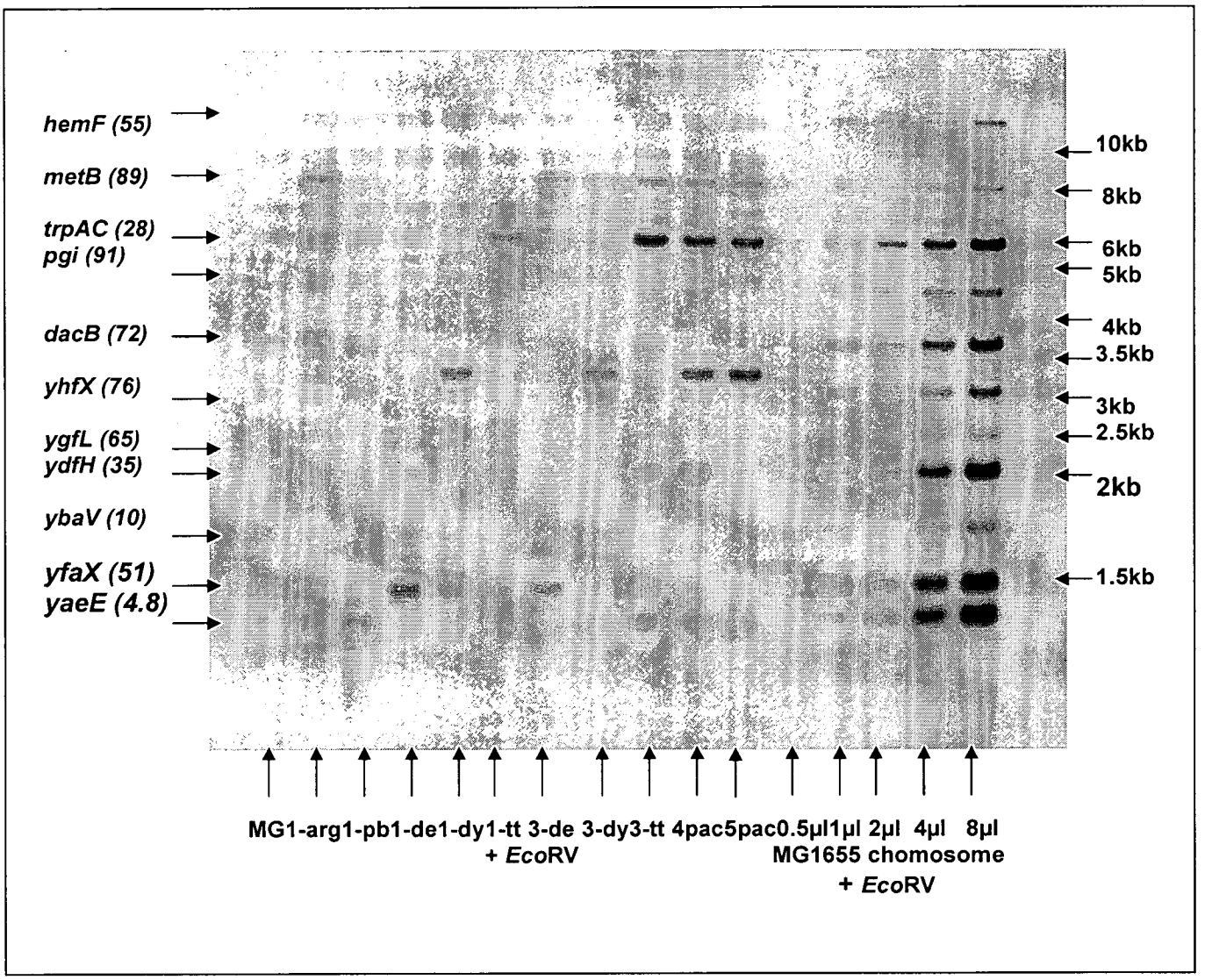


Fig 4.34 Analysis of the chromosomal packaging of multi-*pac* strains using Southern blot (26/02/03).

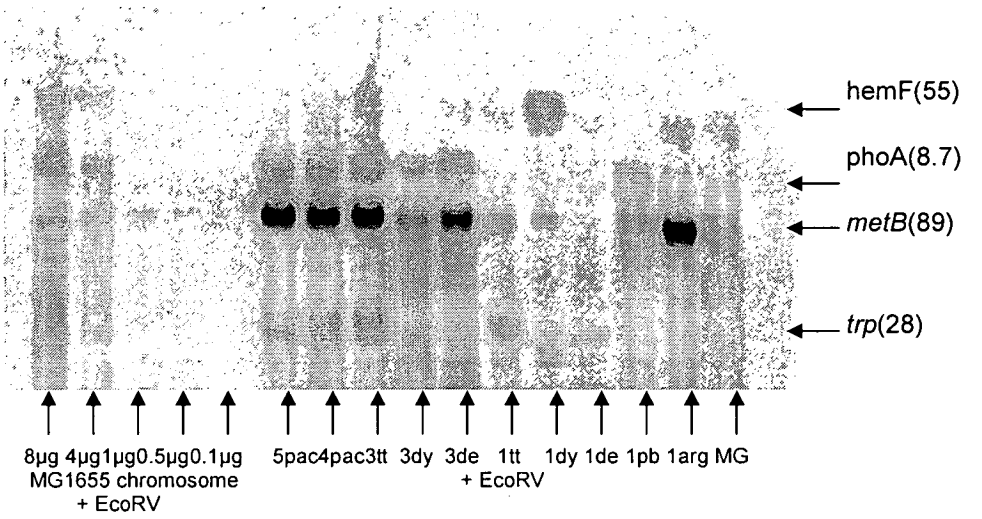


Fig 4.35 Test of the packaging of marker phoA in multi-*pac* strains by Southern blotting.

The packaging ratio (signal from band in experimental-strain P1 DNA/ signal from corresponding MG1655-originating P1 DNA) was calculated for each band in each experiment, and is listed in Table 4.15 and plotted in Fig 4.36. We can see that the chromosomal packaging patterns of different *pac* strains are similar to what we expected except that 5pac shows no evidence of packaging from *pac*(de). However, multiple *pac* sites do not appear to increase the packaging frequencies of markers along the entire chromosome; rather, it appears that packaging peaks and troughs are formed around the *pac* sites. Therefore, the effects of multiple *pac* sites on the chromosome are not additive. How packaging is carried out when multiple *pac* sites are present on the chromosome is not clear. One possibility is that only one or a few *pac* sites are used for packaging in each individual cell, which will probably result in some increase in the total amount of DNA packaged. Alternatively all *pac* sites may be used in each cell with a concomitant increase in packaging of some markers and degradation of others. Again we cannot predict whether total DNA packaged will be increased or by how much. Further experimental work is required to further understand how the packaging of chromosomal DNA proceeds in multi-*pac* strains.

Table 4.15 The packaging ratio (signal from band in experimental-strain P1 DNA/ signal from corresponding MG1655-originating P1 DNA).

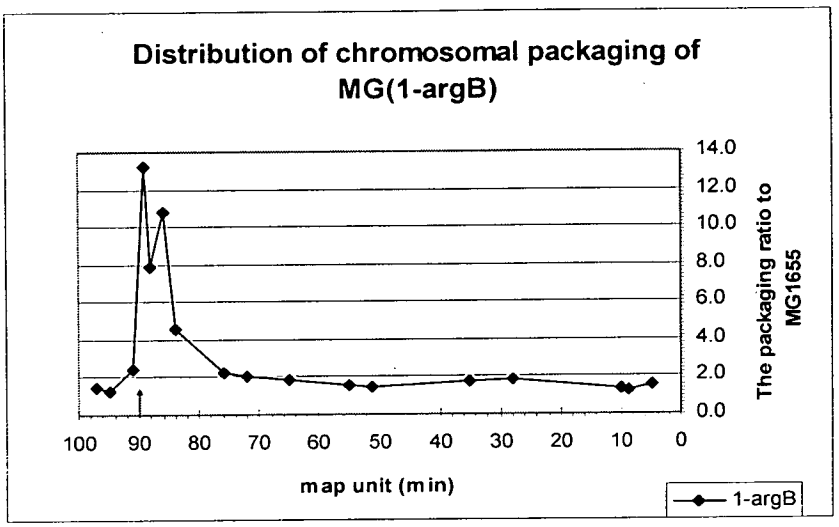
	MG	1-argB	1-pb	1-de	1-dy	1-tt	3-de	3-dy	3-tt	4pac	5pac	Min on the map	*pac* position
97	3247	4619	4093	1805	2090	3549	1988	2345	2072	2062	1282	97	
95	2203	2699	2643	2114	2356	2610	2204	3230	1590	1465	1197	95	
1	867.7	2141	1325	942.7	1495	2179	1595	1180	2090	2144	1245	91	
	1859	22653	1298	1187	1438	1951	17434	11517	26855	21700	31963		*pac*<>arg
3 89	585.6	8321	1044	769.2	1134	1032	6621	4131	9421	7269	10158	89	(89)
88	4365	34583	3357	2150	2784	4901	40879	34583	50693	47082	62860	88	
D	4797	51798	4143	1080	2740	4710	23142	23438	32799	28662	36752	86	
84	3695	16907	1363	1784	2021	1693	6491	4493	8849	6388	7327	84	
	3117	7301	2492	1792	9988	2967	2158	18036	3107	10842	8632	76	
76	1430	2909	1698	1228	2299	2026	1486	2124	1749	2415	2608	76	
	4929	11439	4330	2549	36547	4407	2061	44654	6577	68216	91255		*pac*=>dy
3 72	2258	4001	1998	1430	19633	2791	1693	17873	2635	25214	33038	72	(72)
65	813.3	1538	1634	515.3	2163	1374	970.2	1505	1143	1154	939.7	65	
F55	1180	1802	1590	2036	3526	2110	1546	1203	2264	2622	2529	55	
51	3322	4726	3420	22066	5612	3001	15065	1645	2638	3048	1666	51	*pac*=>de (53)
35	2131	3653	1935	4855	1560	2150	1412	1353	2238	2964	1328	35	
	3045	4926	2340	5722	2896	18262	2346	3052	52913	57490	71883		*pac*=>tt
©28	1605	3374	1791	3545	2531	15280	2060	1695	42443	32834	32156	28	(30)
	3420	5538	3294	2027	2435	4198	3225	5944	2213	2880	3113		
/ 10	1741	1876	2113	282	1511	1768	1421	1288	1337	1121	717.6	10	
A8.9	2077	2727	6952	1905	693	1386	11614	5207	15155	14512	13888	8.9	*pac*=>pb (9.0)
E4.8	3421	5486	13792	2136	2611	2591	5970	4417	6542	6498	5730	4.8	

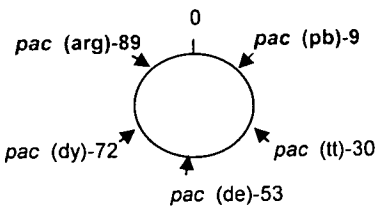
A

Makers + location	Densities of P1 DNA from different strains (normalized by P1 (MG1655) density										
	MG	1-arg	1-pb	1-de	1-dy	1-tt	3-de	3-dy	3-tt	4pac	5pac
holC 97	1	1.4	1.3	0.6	0.6	1.1	0.6	0.7	0.6	0.6	0.4
yjfJ 95	1	1.2	1.2	1.0	1.1	1.2	1.0	1.5	0.7	0.7	0.5
pgi 91	1	2.5	1.5	1.1	1.7	2.5	1.8	1.4	2.4	2.5	1.4
metB 89	1	**13.2**	1.2	1.0	1.4	1.4	**10.3**	**6.6**	**15.3**	**12.0**	**17.3**
yihU 88	1	7.9	0.8	0.5	0.6	1.1	9.4	7.9	11.6	10.8	14.4
hemD 86	1	10.8	0.9	0.2	0.6	1.0	4.8	4.9	6.8	6.0	7.7
atpG 84	1	4.6	0.4	0.5	0.5	0.5	1.8	1.2	2.4	1.7	2.0
yhfX 76	1	2.2	1.0	0.7	2.4	1.2	0.9	3.6	1.1	2.6	2.3
dacB 72	1	2.0	0.9	0.6	**8.1**	1.1	0.6	**8.5**	1.3	**12.5**	**16.6**
ygfL 65	1	1.9	2.0	0.6	2.7	1.7	1.2	1.9	1.4	1.4	1.2
hemF 55	1	1.5	1.3	1.7	3.0	1.8	1.3	1.0	1.9	2.2	2.1
yfaX 51	1	1.4	1.0	**6.6**	1.7	0.9	**4.5**	0.5	0.8	0.9	**0.5**
ydfH 35	1	1.7	0.9	2.3	0.7	1.0	0.7	0.6	1.1	1.4	0.6
trpA© 28	1	1.9	0.9	2.0	1.3	**7.8**	1.0	1.0	**21.9**	**19.7**	**21.8**
ybaV 10	1	1.3	1.1	0.4	0.8	1.1	0.9	1.2	0.7	0.7	0.7
phoA 8.7	1	1.3	**3.3**	0.9	0.3	0.7	**5.6**	**2.5**	**7.3**	**7.0**	**6.7**
yaeE 4.8	1	1.6	4.0	0.6	0.8	0.8	1.7	1.3	1.9	1.9	1.7

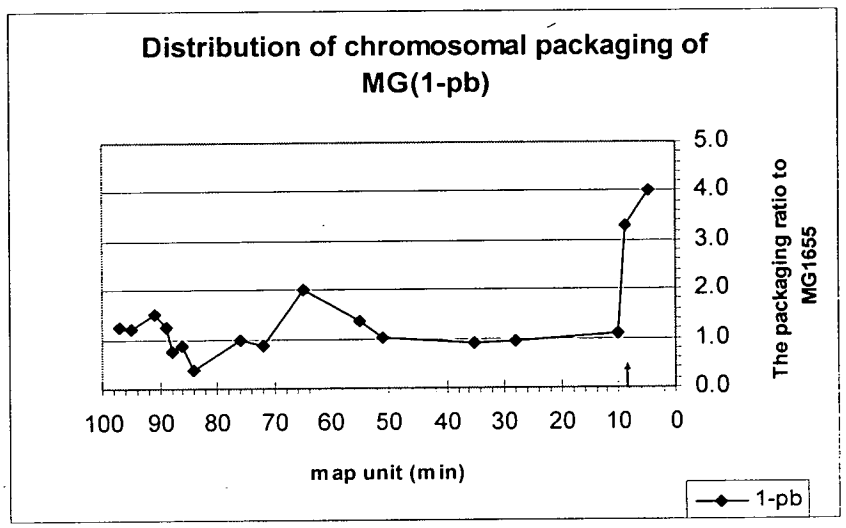
B

Note: **A**) the original area values for bands of each marker. The position of the *pac* sites has been shown next to the marker which is closest to this *pac site*. Some markers, such as *metB*, *trpA*, *dacB*, *yhfX* and *ybaV*, have been measured in both Southern blottings and their data from both experiments are listed in the Table A. The average packaging ratios for these markers have been calculated and listed in Table B. **B**) the packaging ratios of each marker from MG1655 to the same marker from different *pac*-containing strains. Numbers in bold are the packaging ratios of markers closest to the corresponding *pac* site.

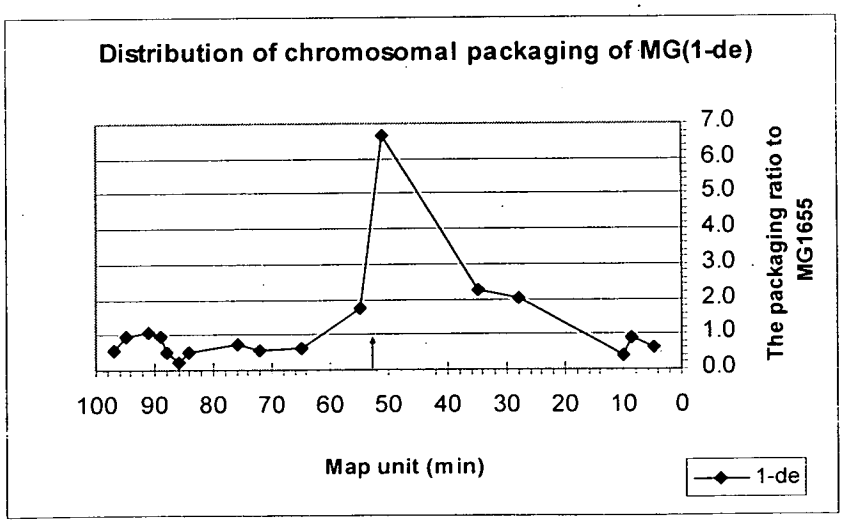

Distribution of chromosomal packaging of MG(1-argB)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
map unit (min)
1-argB


0
pac (arg)-89
pac (pb)-9
pac (dy)-72
pac (tt)-30
pac (de)-53

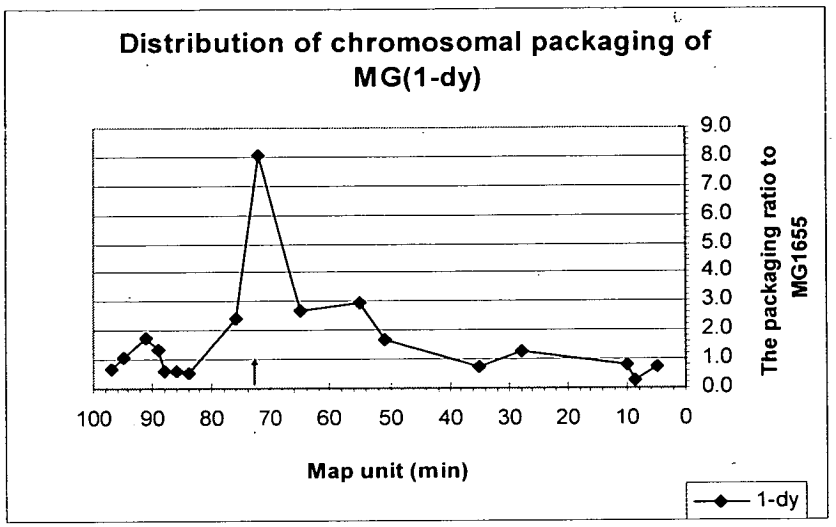
A

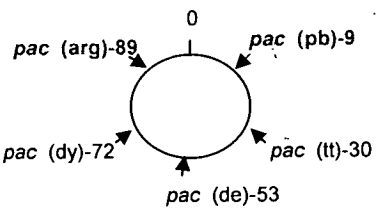

Distribution of chromosomal packaging of MG(1-pb)
5.0
4.0
3.0
2.0
1.0
0.0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
map unit (min)
1-pb

B

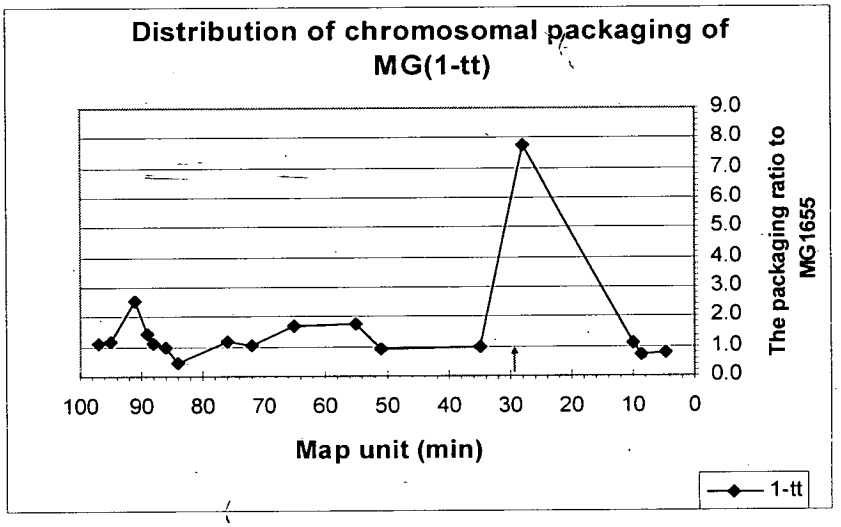

Distribution of chromosomal packaging of MG(1-de)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
Map unit (min)
1-de

C

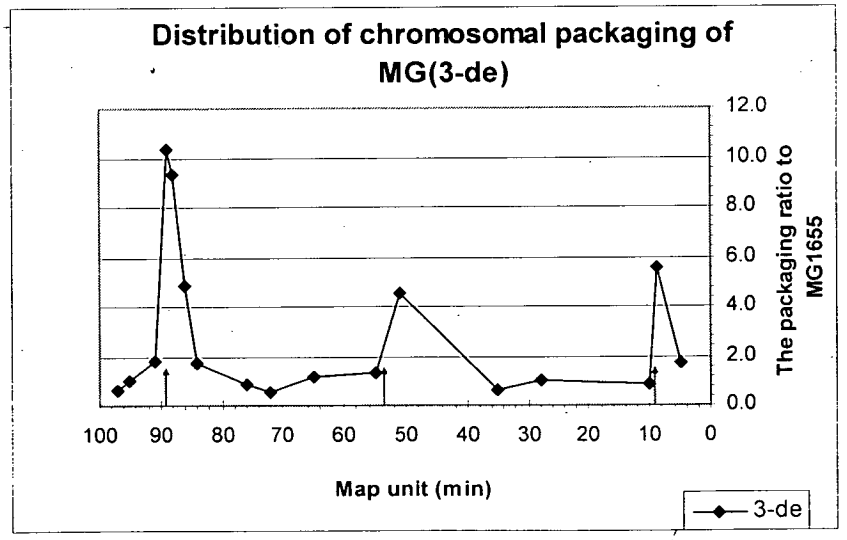

Distribution of chromosomal packaging of MG(1-dy)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
Map unit (min)
1-dy


0
pac (arg)-89
pac (pb)-9
pac (dy)-72
pac (tt)-30
pac (de)-53

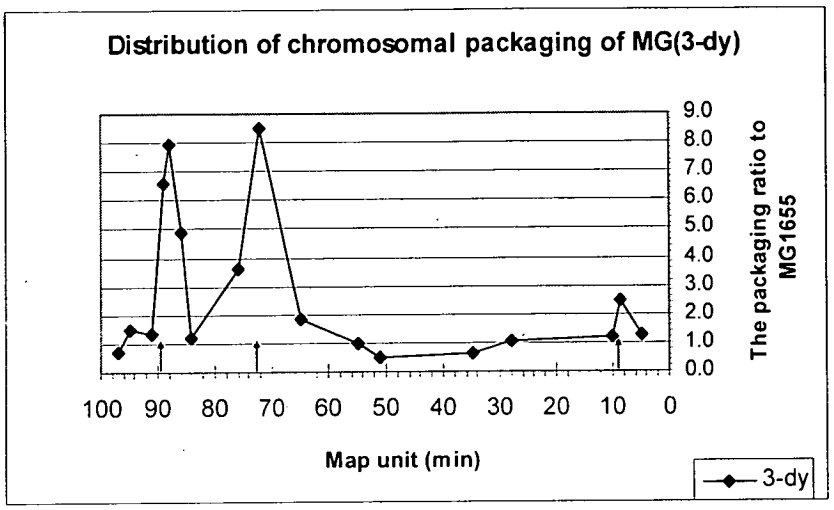
D

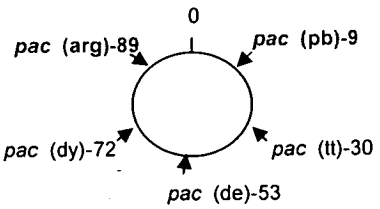

Distribution of chromosomal packaging of MG(1-tt)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
Map unit (min)
1-tt

E

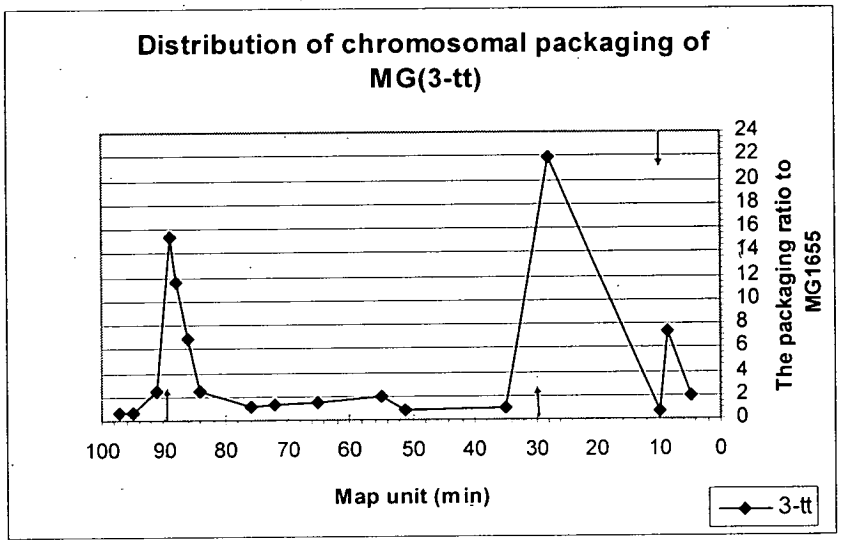

Distribution of chromosomal packaging of MG(3-de)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
Map unit (min)
3-de

F

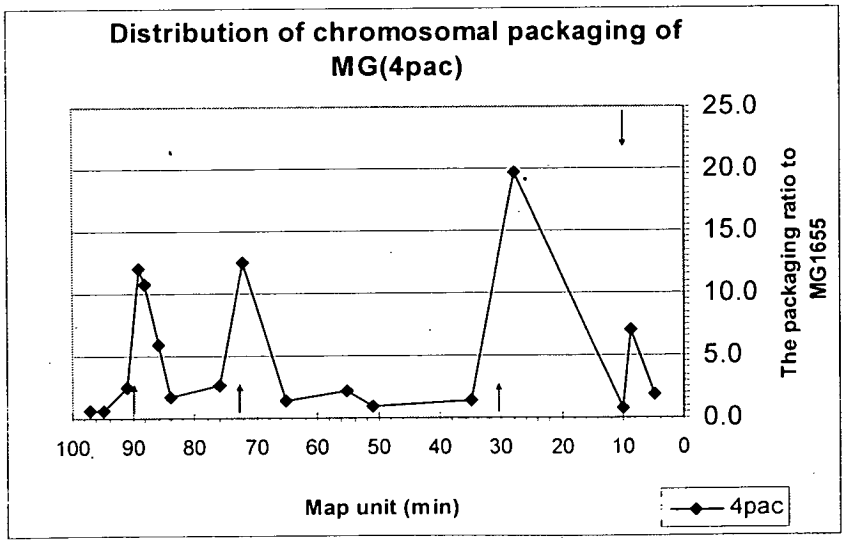

Distribution of chromosomal packaging of MG(3-dy)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
Map unit (min)
3-dy


0
pac (arg)-89
pac (pb)-9
pac (dy)-72
pac (tt)-30
pac (de)-53

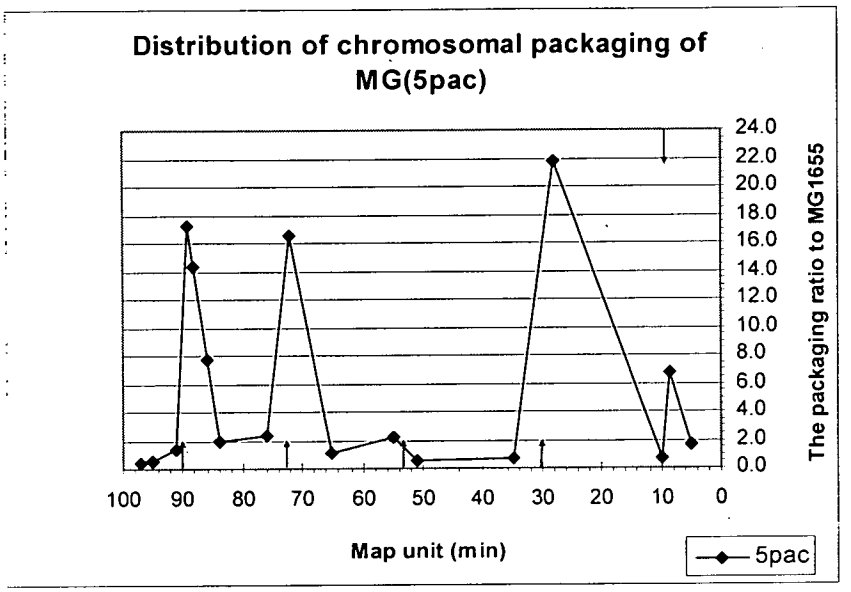
G

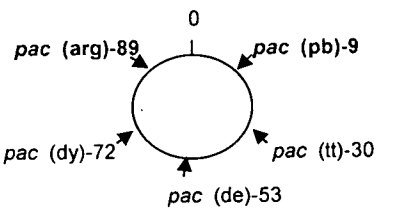

Distribution of chromosomal packaging of MG(3-tt)
24
22
20
18
16
14
12
10
8
6
4
2
0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
Map unit (min)
3-tt

H


Distribution of chromosomal packaging of MG(4pac)
25.0
20.0
15.0
10.0
5.0
0.0
The packaging ratio to MG1655
100 90 80 70 60 50 40 30 20 10 0
Map unit (min)
4pac

I





J

Fig 4.36 Distributions of the chromosomal packaging of different *pac*-strains. 5 *pac* positions are shown in the mini map. Since all strains which have 2 or more *pac* sites contain *pac* (argB) and *pac* (pb) (see Fig 4.17 flow sheet), the arrows in bold show the positions of these two *pac* sites. For *pac*(de) in 5pac graph, please see detail in the text and refer to Fig 4.34 and Table 4.14 below.

4.3.7 Do *pac* sites on the chromosome interfere with each other in packaging the chromosomal DNA?

The next experiment addresses the question of whether or not more *pac* sites on the chromosome lead to greater chromosomal DNA packaging. Three P1 lysates, P1(1-argB), P1(3-dy) and P1(5pac) were used (this is because most available markers are located between *pac*(argB) and *pac*(dy)) and the packaging ratios of markers between *pac* located at 89 min and *pac* located at 72min was measured (Fig 4.37). A Southern blot was performed and data analyzed as before. The packaging ratios of markers from *pac*-containing strains to that from MG1655 were calculated, listed in table 4.16 and plotted in Fig 4.38.

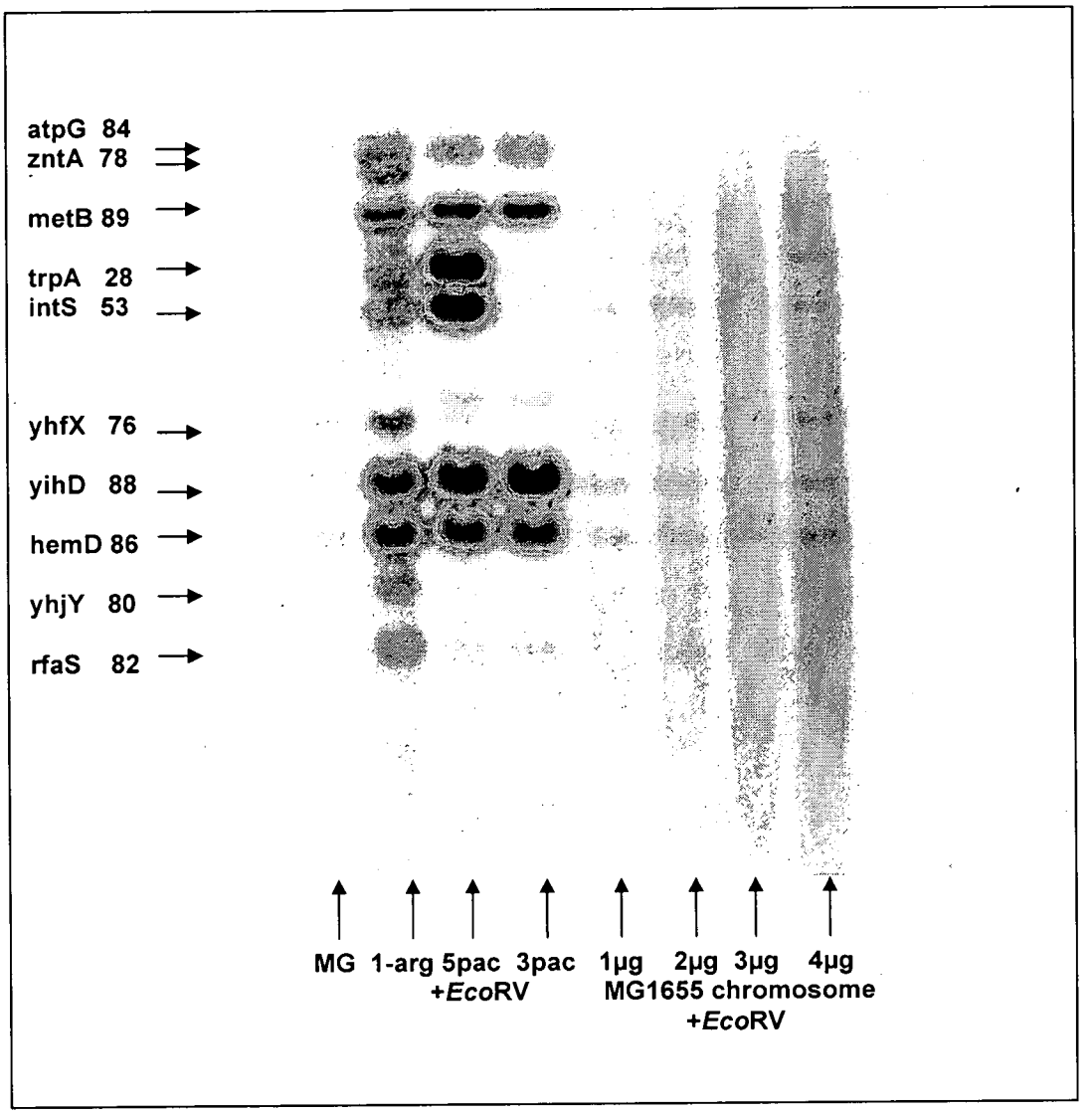


Fig 4.37 Examination of interaction between *pacs* in multi-*pac* strains. 6.2 µg of each P1 DNA was loaded on the agarose gel after *Eco*RV digestion. The P1(MG), P1(3-dy) and P1(5pac) DNA were newly made.

Table 4.16 Analysis of the packaging of markers located between 89min *pac* and 72min *pac*.

	MG	1-arg	5pac	Ratio of 5pac to1-arg	3-dy	Ratio of 3-dy to1-arg	min on the map
metB 89	1	**35.3**	**56.1**	1.6	**58.4**	1.7	89
yihU 88	1	**30.2**	**56.2**	1.9	**61.5**	2.0	88
hemD 86	1	**39.7**	**33.7**	0.8	**39.9**	1.0	86
atpG 84	1	**10.7**	**6.4**	0.6	**7.4**	0.7	84
rfaS 82	1	**21.8**	**3.6**	0.2	**3.9**	0.2	82
yhjY 80	1	**21.2**	**3.2**	0.2	**2.2**	0.1	80
zntA 78	1	**10.8**	**1.5**	0.1	**1.4**	0.1	78
yhfX 76	1	**5.2**	**1.2**	0.2	**0.9**	0.2	76
intS 53	1	**8.3**	**146.5**	17.7	**1.1**	0.1	53
trpA© 28	1	**6.5**	**105.9**	16.3	**0.8**	0.1	28

Note: markers, *intS* and *trpA*, were used to verify the strain MG1655 (5pac). Details about use of *intS* and *trpA* in this work are described in text.

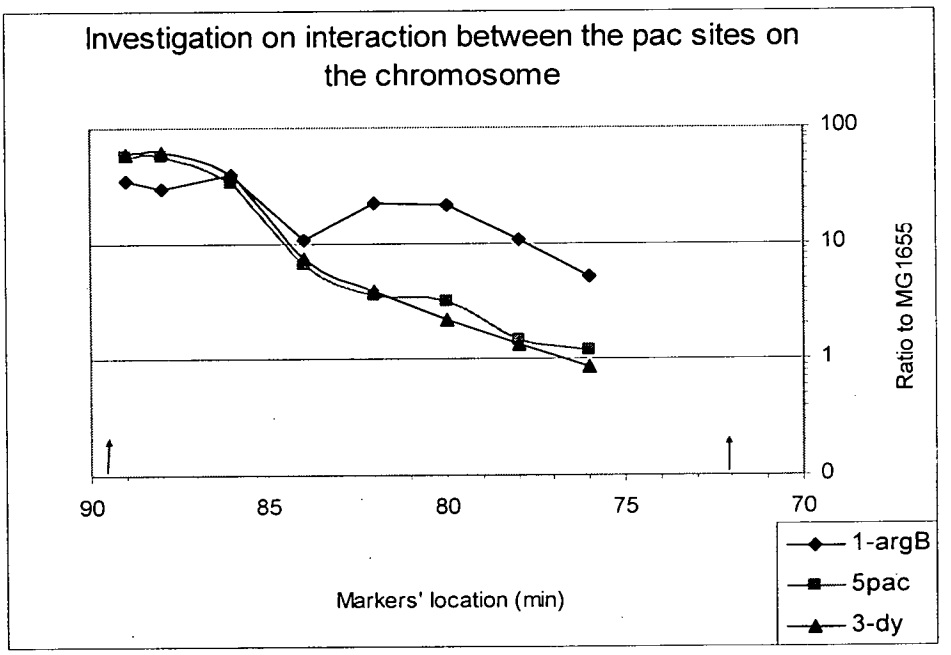


Fig 4.38 Distribution of the packaging of markers located between *pac* located at 89min and *pac* located at 72 min to detect interaction between the *pac sites* on the same chromosome.

pac sites are located at 89min and 72min in 3-dy and 5pac. The packaging ratio of markers located behind the second *pac* is much reduced relative to that of the same markers in the single *pac* strain, MG1655(1-arg) (also see southern blots for studying single-*pac* strains above), This finding can be explained by the fact that the non-packaged end generated by *pac* cleavage is rapidly degraded. In a multi-*pac* strain, there are opposing effects, one which increases, the other which decreases packaging. It is thus difficult without an alternative means of analysis to determine whether the total amount of transducing DNA packaged is raised by integrating several *pac* sites into the chromosome.

In this experiment, packaging of all markers in the single *pac* strain appears to be increased relative to the MG1655 lysate, even that of *trpA* which is 60min from *pac*. Since it seems unlikely that the processivity of packaging can be this great, it is possible that packaging in this MG1655 lysate was low or DNA concentration incorrectly estimated. It is thus not possible to definitely distinguish between increased packaging of markers distant from *pac* in the single *pac* strain relative to MG1655 and decreased packaging relative to MG1655 by the multi-*pac* strains.

The markers *intS* and *trpA* were tested here to verify the activity of the *pac* sites de and tt in MG1655 (5pac), due to the previous observation that the packaging rate of *yfaX* appeared not to be increased from MG1655 (5pac). The possibility that *pac*, located at 53.4, may not be functional is ruled out by this Southern result, since the packaging of *intS*, which is located at 53.2min on the coli-chromosomal map, was greatly increased. The reason why the packaging of *yfaX*, located at 51min, was barely affected by *pac* from 5pac strain is not clear in the previous experiment. It cannot be rule out that I may make a mistake by taking 4pac strain twice or the 5pac strain I used for this experiment has mutated since the culture I used was old.

Because of the consideration above and to confirm the above result, another Southern blot was performed using additional P1 lysates (Table 4.17, Fig 4.39). 11 markers in total were examined, in which 8 markers are located between *pac* (argB) 89min and *pac* (dy) 72min, while 3 other markers are located far away from the above two *pac* sites. Ratio of packaging of each marker in each lysate relative to that of P1(MG1655) DNA was calculated. From Fig 4.40, we can see that markers close to *pac* (dy), 72min, such as *rfaS* and *yhjY*, in P1(3-tt) DNA were packaged about 7 fold higher than in P1(3-dy) and P1(5pac) were. Since the amount of P1(2pac) DNA loaded on the agarose gel is low, the

result for P1(2pac) may not be reliable. Also, from Fig 4.40, reduction of packaging of markers behind *pac*(dy) for multi-*pac* strains is not apparent relative to the result in Fig 4.37. One problem is that there are not markers which are close enough to *pac*(dy). More markers around 72min and behind *pac*(dy) are required in the future work. The result of this experiment indicates that even though reduction of packaging of markers on the other side of the *pac* site is observed, the reduction is too low to counteract the increase of packaging resulting from the *pac* site. Therefore, the total packaged chromosomal DNA by P1 is increased by the inserted *pac* sites.

Table 4.17 Analysis of the packaging of markers between *pac* sites.

Markers	min	P1(MG)	P1(1-de)	P1(1-dy)	P1(2pac)	P1(3-dy)	P1(3-tt) *	P1(5pac)
metB	89	1	1.3	4.7	61.2	62.6	38.2	65.6
yihU	88	1	1.2	1.5	84.2	89.4	67.7	104.2
hemD	86	1	0.5	1.0	24.3	31.1	31.9	34.7
atpG	84	1	0.6	1.2	9.2	5.5	2.7	4.4
rfaS #	82	1	1.4	1.3	8.1	6.8	14.2	4.7
yhjY #	80	1	1.9	1.3	6.5	2.2	10.2	2.8
zntA	77	1	2.2	1.4	1.1	1.1	0.4	1.5
yhfX	76	1	1.3	2.0	1.5	2.9	1.8	3.3
*trp*A,C	28	1	6.4	2.0	1.8	1.3	84.1	100.2
pstG	25	1	5.1	1.8	2.1	0.1	17.2	17.7
yleB	15	1	1.8	1.7	0.9	0.5	4.1	0.7

Note: packaging ratio of each marker in different P1 DNA to that in P1(MG1655) DNA was calculated using Imagequant as described above. *: since there is a strong background for P1(3-tt), the data presented in the table for this lane has been corrected by subtracting the background. #: since the signals for these two markers on the filter are so low, the data in the table for these two markers may not be reliable.

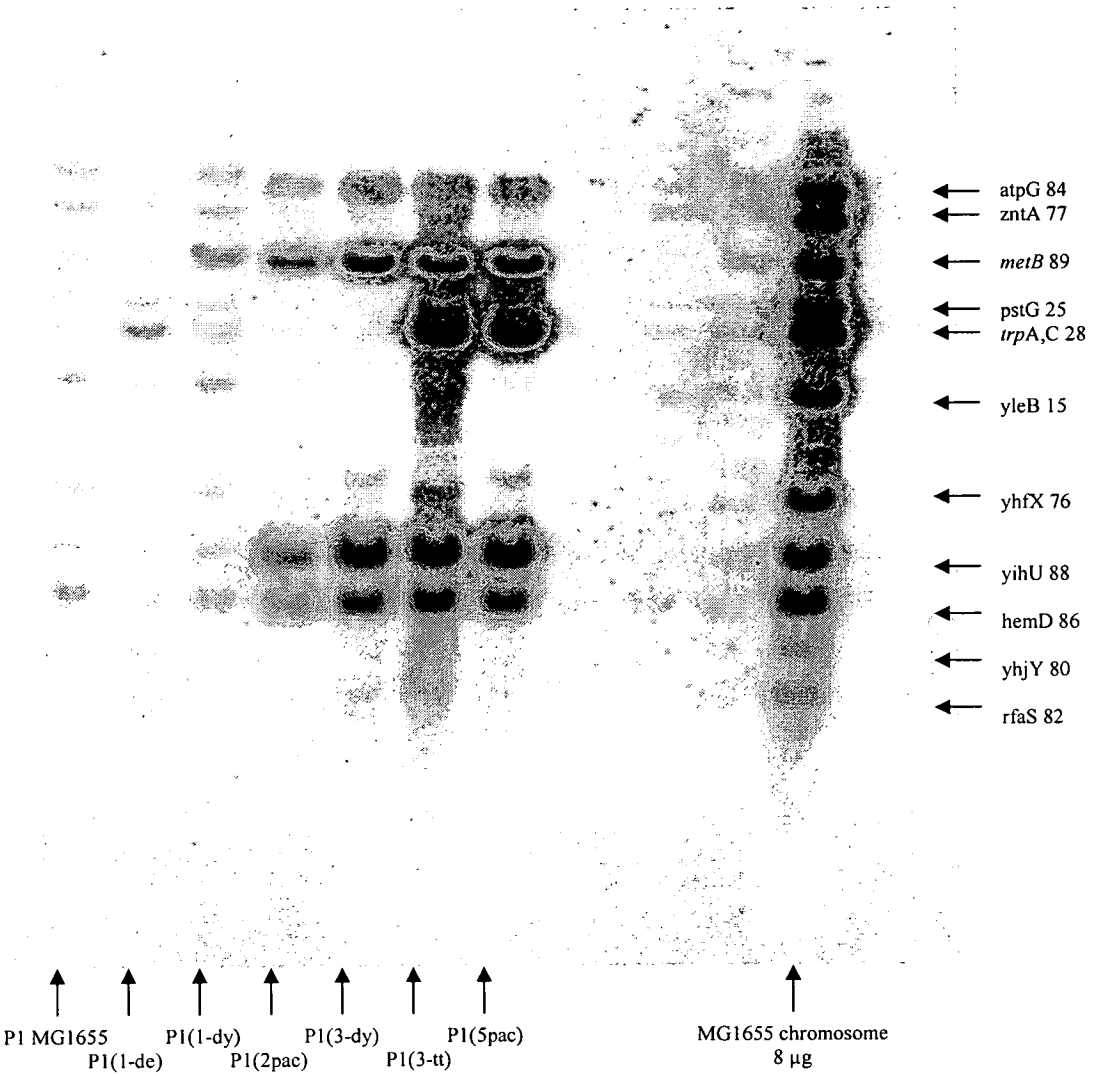
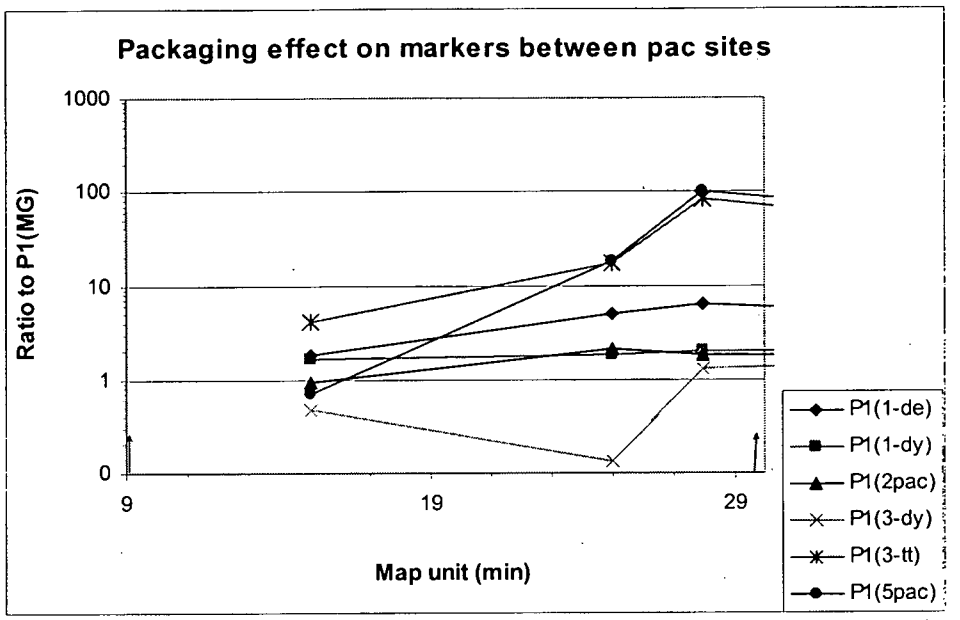
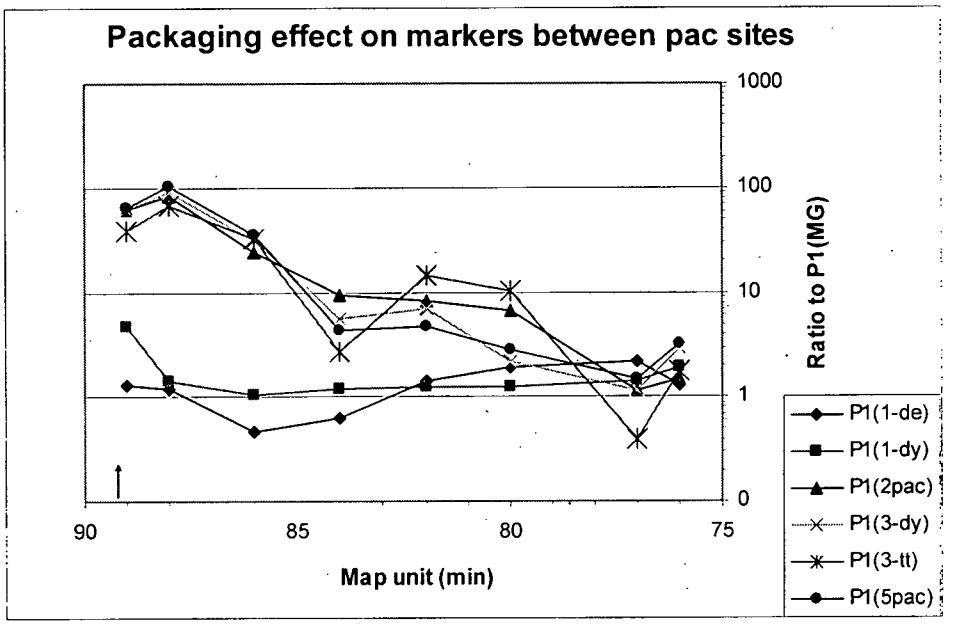


Fig 4.39 Southern blotting to examine interaction between *pacs* in multi-*pac* strains. P1 DNA was extracted from different P1 lysates and loaded on the agarose gel after *Eco*RV digestion. 11µg MG1655 DNA, 4µg P1(1-de), 9µg P1(1-dy), 2µg P1(2pac), 6µg P1(3-dy), 8µg P1(3-tt) and 6µg P1(5pac) DNA were loaded. Southern blot was carried out as described in Materials and Methods.



A



B

Fig 4.40 11 markers in total were examined in the Southern blot (Fig 4.36). 8 markers chosen are located between *pac* (argB) 89min and *pac* (dy) 72min, while other 3 markers are located far away from both of the above *pac* sites. Ratio of packaging of each marker in different P1 DNA to that of P1(MG1655) DNA was calculated. **A)** Distribution of packaging ratio of all markers examined on the chromosome. **B)** Distribution of packaging ratio to P1(MG) of markers between *pac* (argB) and *pac* (dy) in order to see interaction between *pac* sites.

4.3.8 Southern blotting to investigate total chromosomal DNA packaged by P1 from donors containing multiple *pac* sites.

Our original reason for inserting a *pac* site on the chromosome was to increase the amount of transducing DNA packaged by P1. Although we now know that markers close to the *pac* site will be preferentially packaged, we are not clear as to whether more chromosomal DNA in total has been packaged by P1 from *pac*-containing strains.

To answer this question, Southern blots were performed as described above and the High Prime kit (Roche Applied Science, Cat. No. 1 585 592) was used to make probes. High prime is a specially developed reaction mixture containing random oligonucleotides, Klenow polymerase, dATP, dGTP, dTTP, in an optimized reaction buffer. Labelled probes generated with this random primed labelling technique are on average 80-120bp in length, irrespective of the size of input DNA. The principle of this experiment is that when using chromosomal DNA as a template, random priming will generate radioactive labelled probes covering all sequences of the chromosome and thus when using these probes, we should be able to measure the total transducing DNA packaged by P1.

P1 DNAs purified from various P1 lysates were loaded on an agarose gel after digestion by EcoRV (Fig 4.41). 3 different concentrations of each DNA sample, including MG1655 chromosomal DNA, were used in order to demonstrate that the method was quantitative. The filter, after autoradiography, is shown in Fig 4.42. Data were obtained using Imagequant and are shown in Table 4.18. From the filter in Fig 4.42, we can see that each lane shows smearing as expected. Some particular bands are also displayed for all DNA samples. This phenomenon is more apparent for P1 DNA samples relative to chromosomal DNA as weaker and stronger bands appear in P1 DNA lanes. In addition, some bands appear specifically in P1 (multi-*pac*) DNA, suggesting that these bands may result from over-packaged chromosomal fragments due to the existence of *pac* sites. The intensity of total DNA in each lane was measured using Imagequant as described above. As expected, for each DNA sample, the intensities appear to be proportional to the DNA amount loaded in each lane (Table 19; Fig 4.43). To confirm the result, the experiment was repeated and both set of data are listed in Table 4.18. From this table, we can make the following observations: 1) more *pac* sites on the chromosome result in more chromosomal DNA packaged by P1; 2) *pac* sites on the chromosome

result in a 3-7 fold increase of total transducing DNA packaged by P1 from that strain relative to that of a *pac*-free strain; 3) the difference in the amount of DNA packaged from single *pac* and multi-*pac* strains is not great; 4) the increase of total chromosomal DNA packaged by P1 is much less than that of markers close to the *pac* sites observed in the previous work. This result is consistent with the result shown in section 4.3.7.

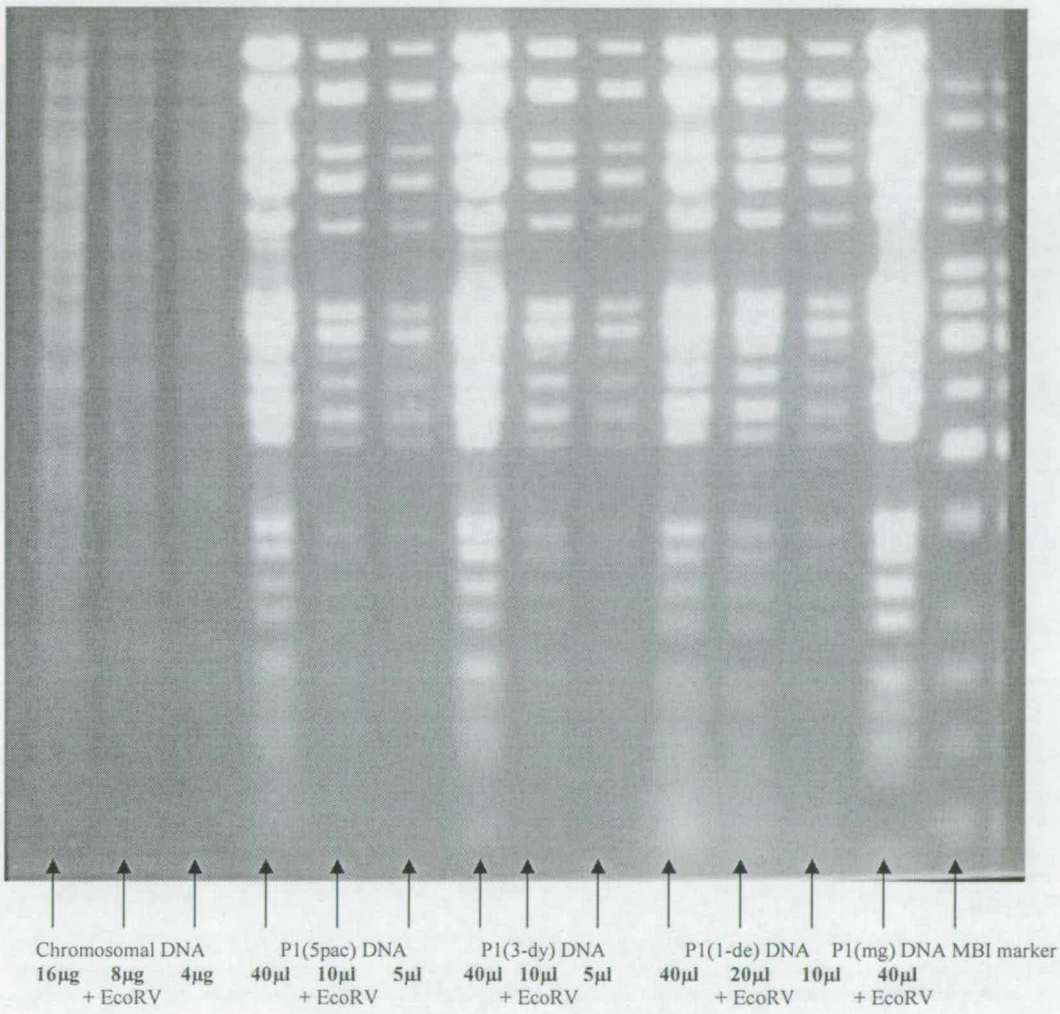
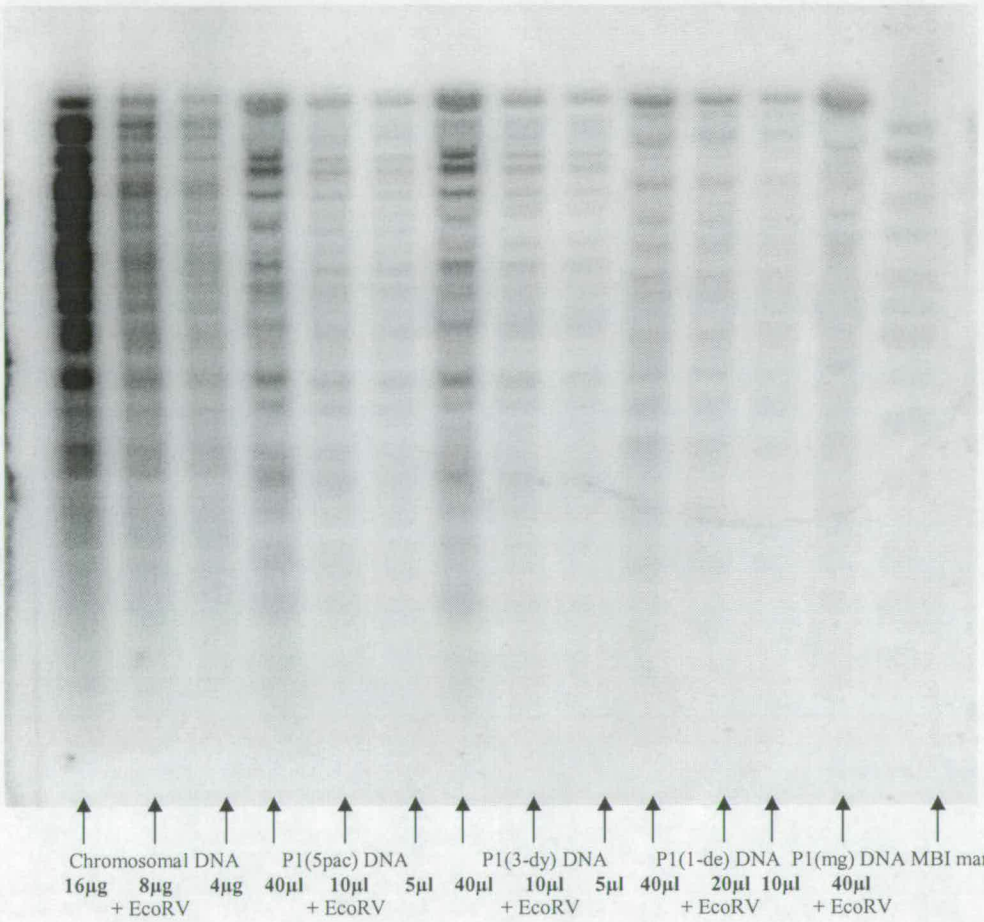
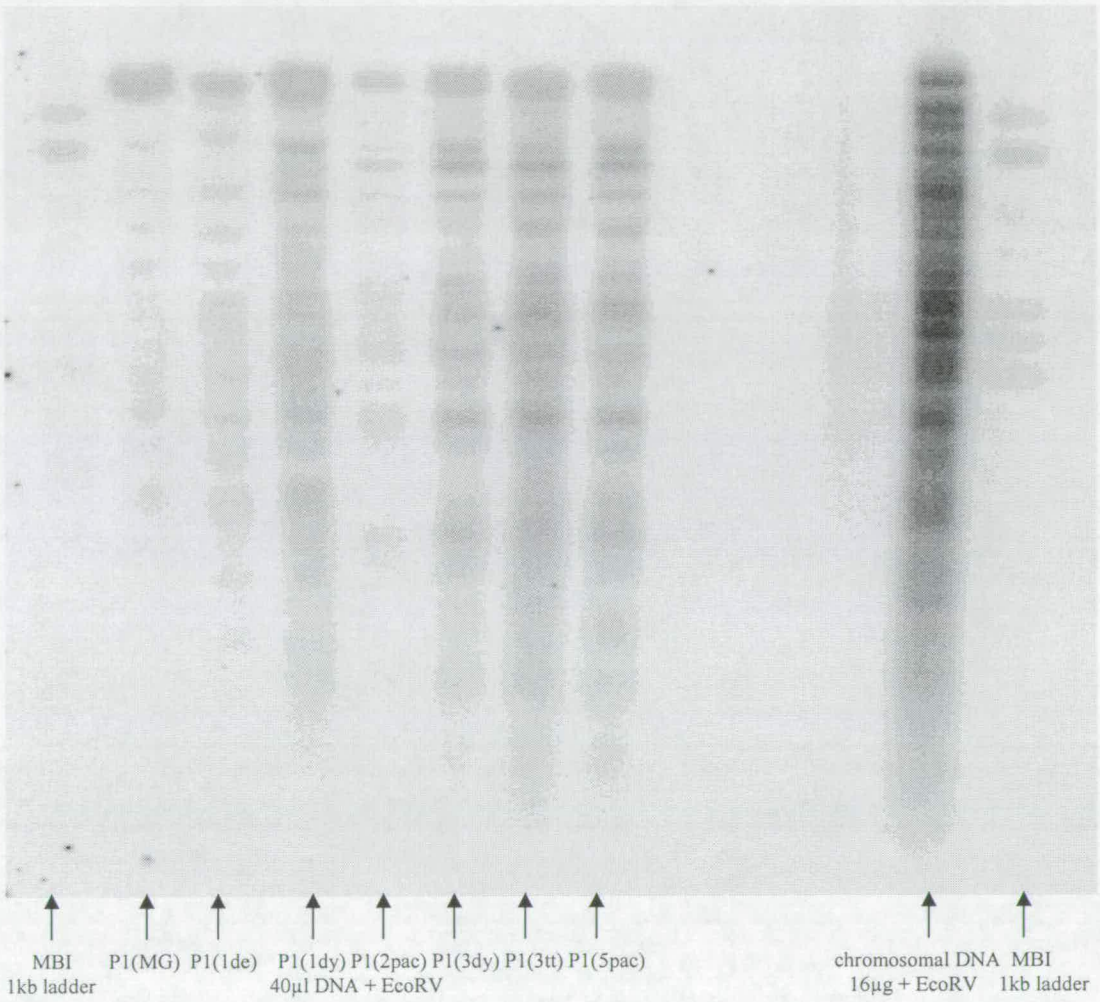


Fig 4.41 P1 DNAs extracted from different P1 lysates were fractioned on an 0.8% agarose gel after digestion by EcoRV. Details are in the text.


Chromosomal DNA
16µg 8µg 4µg
+ EcoRV
P1(5pac) DNA
40µl 10µl 5µl
+ EcoRV
P1(3-dy) DNA
40µl 10µl 5µl
+ EcoRV
P1(1-de) DNA
40µl 20µl 10µl
+ EcoRV
P1(mg) DNA
40µl
+ EcoRV
MBI marker

A



B

Fig 4.42 Examination of packaging total chromosomal DNA by P1 using Southern blottings. **A**) the filter shows the result of 19/11/2003 Southern blotting, in which different concentrations of each DNA sample were examined. Details are in the text. **B**) the filter shows the result of 13/11/2003 Southern blotting. P1 DNA sample each from a variety of P1 lysates were examined.

Table 4.18 Analysis of Southern blots using Imagequant for examination of total packaged chromosomal DNA by P1.

Name of P1 DNA	Area intensity	Amount of DNA (μg)	Ratio to P1(MG)
P1(MG)	131469	11	**1**
P1(1-de)	196879	4	**4.1**
P1(3-dy)	456192	6	**6.4**
P1(5pac)	541332	6	**7.5**

A

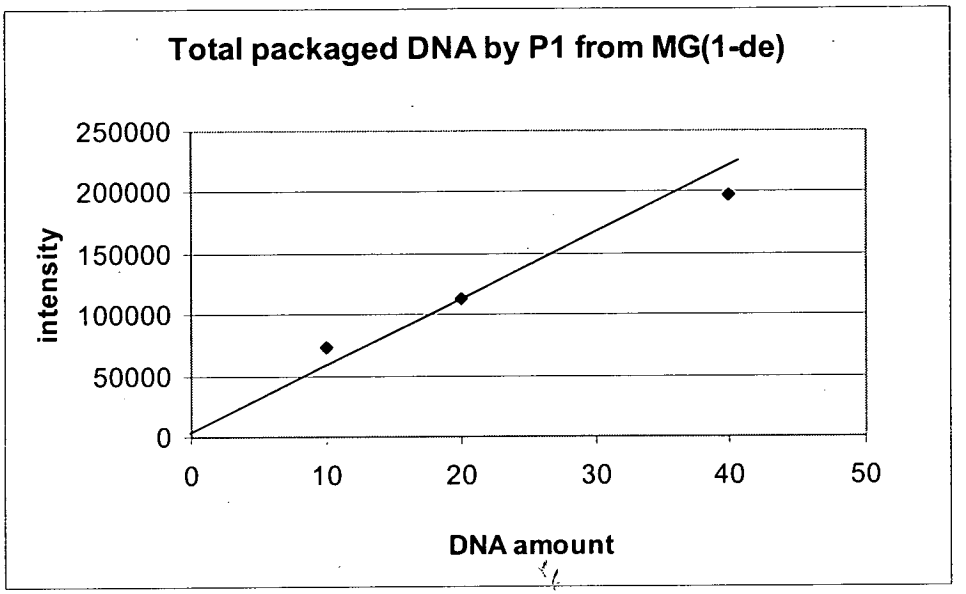
Name of P1 DNA	Area intensity	Amount of DNA (μg)	Ratio to P1(MG)
P1(MG)	10465	11	**1**
P1(1-de)	14315	4	**3.8**
P1(1-dy)	26252	9	**3**
P1(2pac)	8381	2	**4.4**
P1(3-dy)	25316	6	**4.4**
P1(3-tt)	40979	8	**5.4**
P1(5pac)	30771	6	**5.4**

B

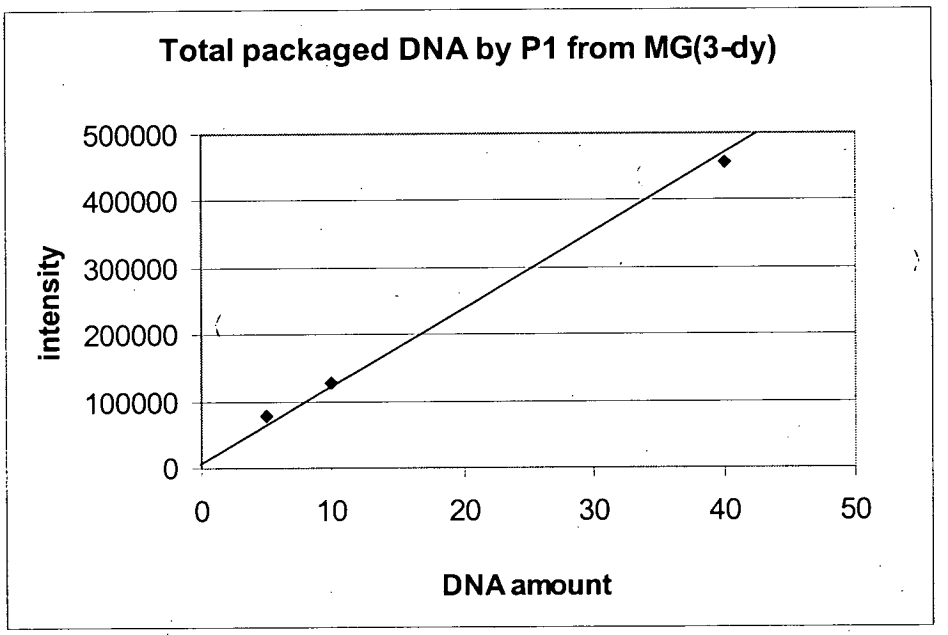
Note: **A**) Data from Fig 4.42-A. **B**) Data from Fig 4.42-B. One possibility that lower intensities were shown in Table B relative to Table A is that lower DNA amount present on the filter of Fig 4.42-B due to lower transferring efficiency as in the work shown in Fig 4.42-B, I transferred two membranes at the same time as described in Molecular Cloning.

Table 4.19 DNA amount of each sample in Fig 4.42-A appears to be proportional to their area intensities measured by Imagequant.

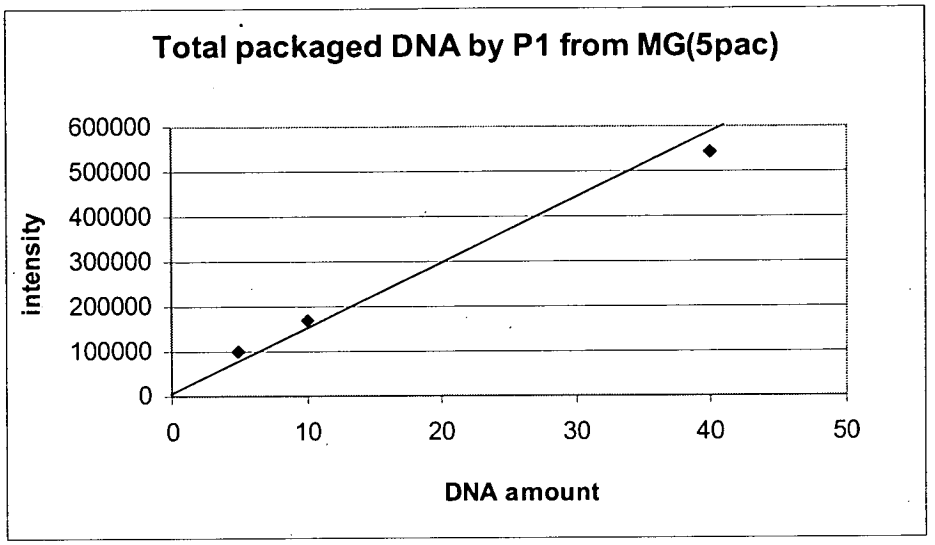
P1(1-de)		**P1(3-dy)**		**P1(5pac)**		**Chrom. DNA**	
DNA volume	band intensity	DNA volume	band intensity	DNA volume	band intensity	DNA volume	band intensity
10 μl	74001	5 μl	79783	5 μl	101683	4 μl	415237
20 μl	113608	10 μl	129333	10 μl	169047	8 μl	745708
40 μl	196879	40 μl	456192	40 μl	541332	16 μl	1746112


Total packaged DNA by P1 from MG(1-de)
250000
200000
150000
100000
50000
0
intensity
0
10
20
30
40
50
DNA amount

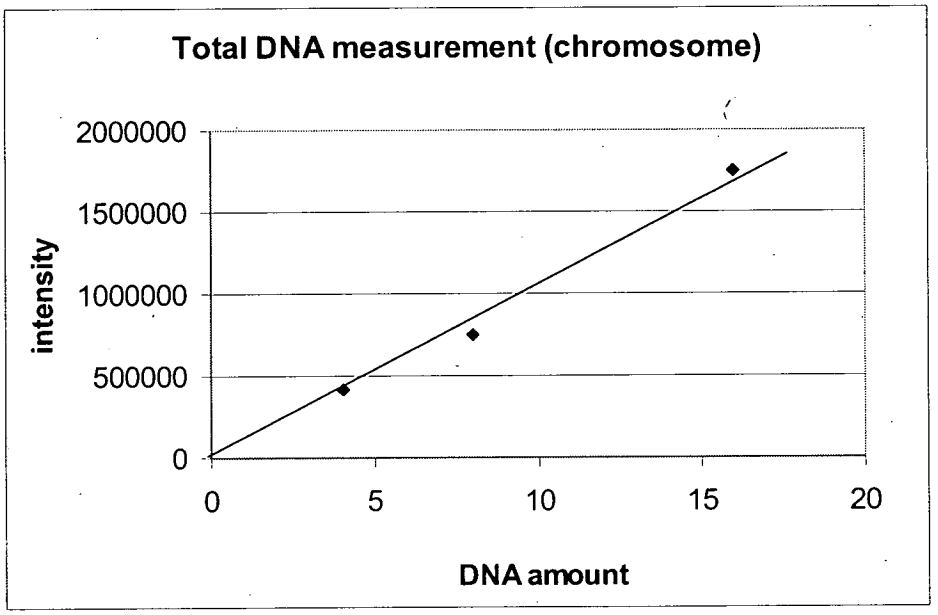
A


Total packaged DNA by P1 from MG(3-dy)
500000
400000
300000
200000
100000
0
intensity
0
10
20
30
40
50
DNA amount

B



C



D

Fig 4.43 DNA amount of each sample in Fig 4.41 appears to be relatively proportional to their area intensities measured by Imagequant.

4.3.9. Discussion and conclusion

4.3.9.1 Packaging of the chromosomal DNA by phage P1

During the vegetative cycle of phage P1, a small proportion of P1 particles do not encapsidate P1 genomes, instead, they encapsidate host chromosomal DNA and therefore are able to transfer genetic material between different strains. The reason why and how the packaging of the host chromosomal DNA is initiated is not clear. One of the possibilities, that *pac*-like sequences are available on the host chromosome, has been ruled out by Hanks *et al.* (1988). He also showed that all genes on the chromosome are packaged at similar levels (Hanks *et al.*, 1988).

To study how P1 packages chromosomal DNA, the P1 packaging site, *pac*, was placed into the chromosome. The packaging from single-*pac* sites and multiple *pac* sites in strains containing up to 5 *pac* sites, was analyzed in this work.

First of all, a *pac* site on the chromosome can greatly facilitate the packaging of markers close to and to one side of it. Transduction experiments showed that a hundred-fold increase in transduction frequency of markers close to a *pac* site can be observed. The maximum increase found in this work is about 10-fold higher than that Sternberg and Coulby (1987a) observed where they inserted a *pac* into the host chromosome within a λ vector. This could be due to the location of the marker and the distance from the marker to the *pac* site, though it cannot be ruled out that placing the *pac* site onto the chromosome without a surrounding replicon is responsible for the differences. Analysis of transduction from multi-*pac* strains showed that each *pac* on a chromosome can be used (see Fig 4.5). At least for the markers nearest *pac*, transduction is similarly elevated in single and multi-*pac* strains. For instance, the transduction frequency of the *metB* marker from MG1655 (5pac) is rather similar to that from MG1655(1-argB) and other markers have a similar increase of hundred times in transduction frequency.

Secondly, UV irradiation experiments showed that the increased transducing particles of particular markers due to the inserted *pac* site probably give rise to more abortively transduced DNA containing

this marker, since the transduction frequency of this portion of newly formed transducing particles is increased by UV irradiation of P1 lysates before infection, suggesting that more stable transductants of a certain marker were formed by converting the increased abortive transductants of the marker via UV irradiation.

The results of Southern blotting confirm that the packaging of the host chromosome initiated from *pac* by Pacase is unidirectional. The end not destined to be packaged has been shown to be rapidly degraded by host nucleases (Sternberg and Coulby, 1987a). This idea results from the observation that the *pac* end destined to be packaged was detected 5 to 20 times more efficiently than was the other end when *pac* was placed on the chromosome, while neither *pac* end was detected efficiently when *pac* was placed on a small plasmid (Sternberg and Coulby, 1987a). We find that markers up to 28 min from *pac* can show increased transduction but that enhanced packaging gradually diminishes with distance from *pac*. Enhanced packaging, although at a low level, was seen to extend farther than Sternberg reported, but the data they used was considerably more limited than our own. The reason why *yihU* and *hemD* were more packaged relative to *metB* is not clear. One possibility is that *metB* is probably too close to the *pac* site. The reason why *atpG* was poorly packaged is as yet unclear. Note that since transducing DNA is only a small portion of P1 DNA, a great deal of P1 DNA and an efficient transfer is required for Southern blots in order to present nice bands after autoradiography. This fact resulted in weak signals for some markers and thus resulted in unreliable data for these markers after analysis using Imagequant. These situations are especially applied to markers with no increased effects by *pac* sites and markers appearing small on the gel.

Southern blots using lysates prepared on multi-*pac* strains indicate that each *pac* on the chromosome containing multiple *pac* sites was able to be used although we cannot say whether more than one is used in any particular cell. Downstream markers near *pac* were packaged at high levels and the effect diminishes with distance from *pac*. It seems that each *pac* functions independently. In contrast to single *pac*-site strains, in a multi *pac* strain, the distance that enhanced packaging extends from each *pac* site is reduced, leading to a series of peaks (downstream from *pac*) and troughs (upstream) in packaging frequency around the chromosome. This pattern probably results from the overlapping of enhanced packaging and degradation regions between *pac* sites, since the multiple *pac* sites placed on the chromosome in this work all are pointing in the same direction. Therefore, it could

be reasonably presumed that if two *pacs* pointing toward each other are placed, an additive effect on the packaging of markers between these two *pacs* could be seen. A question that has been puzzling me since I started my project is whether or not putting multiple *pac* sites onto the chromosome would increase the total number of transducing particles? Results presented in Table 4.16 indicate that although total packaging does appear to be increased in strains with multiple *pac* sites, the magnitude of the increase is not proportional to the number of *pac* sites. A single *pac* site increase packaging four-fold but 5 *pac* sites, placed at equal intervals, do not quite double this packaging ratio.

4.3.9.2. P1 DNA synthesis and possible additional functions of Pacases in the P1 vegetative cycle

As described above, two striking phenomena were observed when titration and lysates of strains containing more than 2 *pac* sites were made. First of all, plate lysate titers of phages made from strains containing more than 2 *pac* sites on the chromosome were reduced 10^3 – 10^4 times relative to those made from a *pac*-free or a single *pac*-containing strain. Secondly, the time when cells containing more than 2 pacsites start to lyse during a single cycle of phage P1 infection is significantly delayed relative to the lysis time for *pac*-free and single *pac*-containing strains. In addition, analysis of P1 DNA concentration during the P1 vegetative cycle revealed that P1 DNA in MG1655 (5-*pac*) accumulated significantly more slowly than P1 DNA in MG1655.

The above results may be telling us that the Pacase plays an essential role not only in P1 packaging but also in the regulation of the P1 growth cycle. This suggests that something essential for continued phage replication and packaging is in limited supply and is titrated by the extra *pac* site of a 2-*pac* strain. The finding that increasing the multiplicity of infection restored a more normal lysis time is consistent with this hypothesis (Fig 4.23). What on earth is that mysterious factor then? PacA protein is a good candidate, since the production of this protein is limited by an inefficient translation mechanism (see Introduction). The yields of the PacA and PacB proteins are very low even using a T7 expression system, as has been reported for other phages. However, replacing the RBS of both genes with the RBS of T7 gene 10 significantly improved the expression of these two genes (Skorupski *et al.* 1992; Skorupski *et al.* 1994a). Furthermore, Skorupski also showed that an 80 to 90-fold molar excess

of PacA relative to *pac* is necessary for normal *pac* cleavage and that the *pac* fragment may have several Pac binding sites, since intermediate binding complexes of PacA and *pac* could form in vitro (Skorupski *et al.* 1994a). Therefore, adding even one *pac* site could consume many molecules of PacA protein.

So, why should limiting the number of PacA molecules not only reduce the yield of infectious phages but also affect phage replication? As mentioned before, Pacase is expressed at an early stage of the viral life cycle. This surprising observation leads us to think that Pacases are not only involved in P1 packaging events, but also have some functions other than packaging. We already know that for many phages, DNA replication switches from circle-to-circle replication (θ) to rolling-circle replication (σ) at late stages of phage growth to produce long concatemers that serve as substrates for packaging of phage DNA into phage heads. However, the molecular basis of regulating the switch from θ to σ replication still remains unknown. Sternberg and Coulby (1990) suggested but did not show that, "Perhaps they (Pacases) play a role in initiating rolling circle replication by nicking circular DNA at *pac*".

From the above results, we presume that the reduction of available Pacase could diminish or delay the initiation of rolling circle replication, due to titration by the *pac* sites on the chromosome. As a consequence the amount of P1 concatemeric DNA formed will be reduced, probably resulting in a decrease in the number of P1 infectious particles. The observation that a poor titer is always obtained from *recA*$^-$ strains, which produce fewer σ replication forms and synthesise less DNA relative to *recA*$^+$ strains is consistent with this hypothesis (Cohen, 1983). Since rolling circle replication may be seriously affected, the number of copies of the P1 genome will be significantly reduced in the cell, at least during the early late period of the growth cycle. Therefore, the synthesis of some late gene products will also be affected. One of these may be the holins (see Introduction). It is thought that the holins control functional access of the endolysin, (a muralytic enzymes that degrades the cell wall), to the murein, by permeabilizing the membrane and thus, the length of the vegetative cycle (Wang, *et al.* 2000). Holins accumulate and oligomerize in the membrane throughout the period of late-gene expression till suddenly triggering a lesion that permeabilizes the membrane (Grundling *et al.* 2001). Therefore, if the production of holins is delayed or the amount reduced, the time of lysis would be delayed as we observed. Results from Southern blots to measure P1 DNA synthesis in infected cells

provide support to the above hypothesis (see, section 4.3.5., Table 4.11, Fig 4.25, Fig 4.26). In MG1655, DNA synthesized reached its maximum about 30 minutes after the completion of adsorption in a synchronous infection system, while in 5-*pac*, it took at least 40 min to approach the maximum. Lysis of MG1655 also occurred sooner and more quickly. In addition, more P1 DNA was also detected in MG1655 than in MG1655 (5pac),

Work on bacteriophage T4 might also be relevant to the above model (Mosig, *et al.* 2001). Two late proteins, endonuclease VII (gp49) and T4 terminase (gp17) appear to be required to create a ss nick to initiate DNA replication. Interestingly, both genes 49 and 17 produce two or more proteins by initiation of translation from internal ribosome binding sites, and the synthesis of the different products is temporally regulated by transcriptional and translational mechanisms (Mosig, *et al.* 2001). The endo VII gene (49) is transcribed early and late from different promoters and produces 2 different proteins, a full-length 18 kDa protein and a shorter 12 kDa protein. The 12 kDa protein is produced early and initiated from an internal GUG, while the 18 kDa protein is produced late since its ribosome binding site is sequestered in a hairpin in the early transcripts. Further work indicated that the 12 kDa homodimers have no endo VII activity, 18 kDa and 12 kDa heterodimers can singly nick and 18 kDa homodimers make concerted ds breaks (Mosig, *et al.* 2001). In this case, remarkably, P1 endonucleases bear an analogy to that of T4. A 20 kDa protein designated PacC is encoded within the C-terminal end of the pac*B* gene and translated in the same reading frame as PacB, while a 42 kDa protein was identified on SDS-polyacrylamide gels and possibly produced from an internal start codon that lies within the pac*A* gene (Skorupski, *et al.* 1992). The role of these proteins still remains unknown. However, this finding suggests a possibility that these P1 proteins might play a role in initiation of P1 replication like their analogues do in the T4 vegetative cycle.

The above results suggest a model in which, in addition to *pac* cleavage, P1 Pacases also possibly play a role in the switch from circle to circle replication to rolling circle replication (Fig 4.27). Perhaps they also play a role in other DNA events involved in nicking or ds break cutting, like recombination or even DNA repair. To test this hypothesis, further work needs to be done. For example, electron microscopy could be used to observe the replication forms in cells as well as other methods.

One question in this model is why the cell harbouring high copy number *pac*-containing plasmids did not result in a low titer of P1 lysates after infection by P1? One possible reason is that since plasmid DNA is rather small relative to a P1 concatemer which is the target for P1 packaging, Pacase binds the *pac* sites on the plasmid and then is released quickly. However, the chromosomal DNA is very long, relative to P1 concatemeric DNA, and therefore, Pacase binds the *pac* sites on it and for some reason, it cannot be released and therefore titrate the concentration of Pacase pool. In addition, how these proteins interact with DNA to achieve their tasks and how the regulation and the timing mechanism in which the P1 pacases are involved works are still unclear. Work using this multi-*pac* system should provide more answers to the above questions. Certainly, since the above mechanism is like a big network, collaborating with pacases, other factors may also be involved in it.

Further work on this multi-*pac* system along with other molecular biological approaches would enable us to understand more about the mechanism of the P1 growth cycle, the regulation of *pac* cleavage, the production mechanism of transducing particles and also the molecular mechanism of the protein-protein interactions and protein-DNA interactions involved in transducing phage formation.

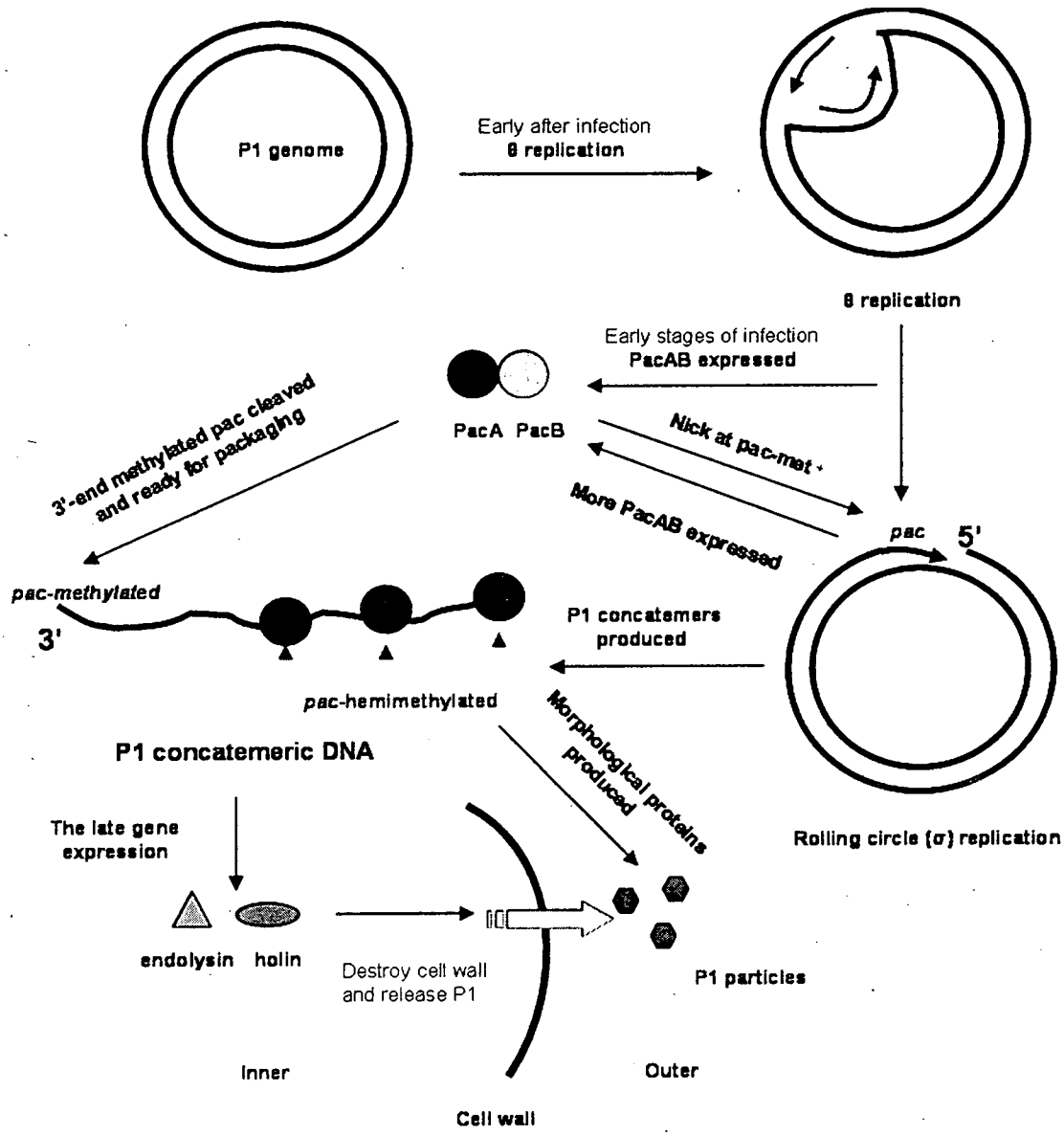


Fig. 4.27 Functions of pacases in P1 growth cycle and packaging. Early after infection, P1 genomes undergo θ replication and a small amount of PacAB is expressed. These PacAB proteins can only stimulate a small number of P1 genomes to undergo σ replication by nicking at the *pac* site. Therefore, there is a similar amount of θ and σ replication during the first 30 min after infection. Later, with increase of PacAB, σ replications dominate the scene after 30 min. During the early late time of infection, PacA proteins bind to the hemi-methylated *pacs* on the P1 concatemers (released 5'-end tails) to protect them from fully methylation and to protect released 5'-end tails from degradation by host nucleases. Once P1 methylases are synthesised and the newly released *pac* is fully methylated, the *pac* is cleaved by PacAB and the rest of the concatemer is ready for packaging. Late after infection, late genes are expressed, including morphological genes and lysis genes. The lysis gene products, holin and endolysin break the cell wall and the new P1 particles are released.

Chapter V

Studies on plasmid transduction by P1

5.1. Introduction

The experiments presented in this Chapter were carried out to study the mechanism of P1-mediated transduction of plasmids. Compared to other well-studied transducing phages (See introduction), more work on plasmid transduction by P1 needs to be done.

O'Connor and Zusman (1983) demonstrated that large plasmids could be transduced by P1 and suggested two possible mechanisms: a P1-plasmid cointegrate model and a multimeric plasmid DNA model (see Introduction). Regarding small plasmids, Iida (Iida *et al.*, 1981) demonstrated that cointegrates of pBR322 derivatives containing IS*1* and P1 DNA (P1 genome contains a IS*1* element) were usually formed by reciprocal recombination between IS*1* sequences, while cointegrates of pBR322 without an IS*1* element and P1 DNA were formed by transpositional cointegration mediated by IS*1* of P1. This event occurs with a frequency 25 times lower than that of reciprocal cointegration. Therefore, Iida and his colleagues (1981) could not observe any transpositional cointegrates unless IS*1* sequences were present on both plasmid and P1 DNA. In addition, Sean Mcteer, in our laboratory, has studied transduction of plasmids carrying chromosomal DNA and found that homology to the chromosome was principally responsible for the P1 transduction of plasmids.

In this work, I studied the effect of homology to P1 DNA on transduction of plasmids and showed that the transduction frequency of plasmids is also increased by homology to the P1 DNA present on plasmids.

5.2 Transduction of plasmids with homology to the P1 genome

5.2.1 Plasmid transduction is enhanced by homology to the P1 genome

MG1655 was transformed with pPac, a pBR325-based plasmid in which the P1EcoRI 21 fragment is cloned. P1 lysates were made from p*Pac* and pBR325-bearing MG1655 strains. Transduction was performed using p*Pac* and pBR325 P1 lysates as donors and the plasmid Amp^+ marker selected in MM303 recipient cells. The result is shown in Table 5.2.

	P1 (pPac)	P1(pBR325)
Titer	3.5×10^{10}/ml	4×10^{10}/ml
Recipient	**MM303**	
Amp^+ transduction freq. (No. of colonies/phage)	**5×10^{-5}**	**5×10^{-8}**
Met transduction freq. (No. of colonies/phage)	**2.3×10^{-6}**	**1.5×10^{-6}**
Recipient	**DH5α**	
Amp^+ transduction freq. (No. of colonies/phage)	**0**	**0**
Met transduction freq. (No. of colonies/phage)		

Table 5.2 Transduction frequencies of Amp+ and Met markers for *recA*$^+$ and *recA* recipient cells by both P1(pPac) and P1(pBR325).

From Table 5.2, we can see that the transduction frequency of p*Pac* is 1000-fold greater than that of pBR325 in a *RecA*$^+$ recipient strain. The *met* marker was chosen as a control to normalize the transduction results. From the above result, it can be concluded that, as observed for transduction of plasmids with homology to chromosomal DNA, homology to the P1 phage genome can also greatly enhance the transduction frequency of markers on the plasmids. Moreover, it is also obvious that the *RecA* protein in the recipient cell is essential for completion of this process, since when using DH5α (*rec*$^-$) as the recipient, no transductants are found on Amp$^+$ plates from either donor.

5.2.2 Enhancement of plasmid transduction is *pac*– independent

Since the P1 EcoRI21 fragment contains the *pac* sequence, we asked whether *pac* plays a specific role in the facilitation of plasmid transduction. To answer this question, another plasmid pProA, containing P1 *proA* gene fragment (ProA protein is putatively involved in phage prohead processing and may also correspond to a protease required for processing of the *darA* precursor protein) was constructed by inserting the P1 *proA* fragment, which is a 650bp PCR product of the P1 *proA* gene, into plasmid pBR325. After transformation, a P1(pProA) lysate was made from MG1655 (pProA). Amp^r was selected after transducing MM303 with P1(pProA) and P1(pPac). The result is shown in Table 5.3.

This result indicates that probably any P1 DNA in the plasmid can increase the transduction frequency of the plasmid, irrespective of the nature of the sequence. In contrast to phage P22, *pac* has no specific role in P1 transduction of plasmids (Schmidt and Schmieger, 1984).

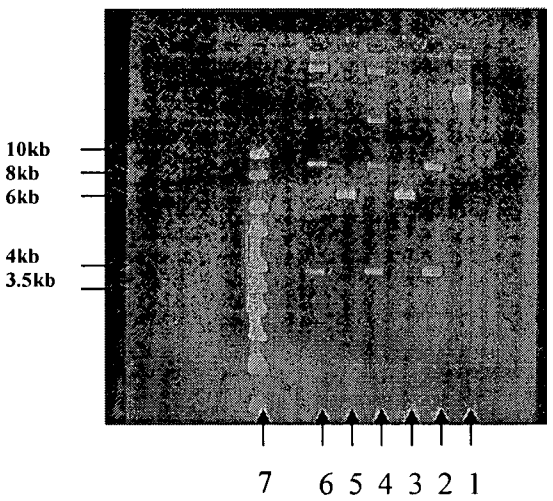
	P1 (pPac)	P1(pProA)
Titer	3.5×10^{10}/ml	2.5×10^{10}/ml
Recipient	**MM303**	
Amp^+ transductants No./ml	**845000**	**1480000**
Amp^+ transduction Freq. (No. of colonies/phage)	**2.4×10^{-5}**	**5.9×10^{-5}**
Met **transductants No./ml** (multiply by 1000)	**43000**	**42000**
Met **transduction Freq.** (No. of colonies/phage)	**1.2×10^{-6}**	**1.7×10^{-6}**

Table 5.3 Transduction frequencies of Amp^R and Met markers by P1(pPac) and P1(pProA).

5.2.3 Nature of the plasmid DNA purified from transductants

To investigate the nature of the plasmid transduced into the recipient cell, plasmid DNA was purified from an overnight culture of one of the transductants and fractionated on a 1% agarose gel (Fig. 5.2). Some very large bands appeared at the top of the gel, close to the well, in Lanes 4 and 6, indicating that plasmid multimers have been formed in the recipient cell. After digestion with BamHI, which only has a single site on the plasmid, the large bands were replaced by a single band, the same size as linearized plasmid, indicating that the large bands seen are purely plasmid DNA only. Note that the plasmids in Lanes 4 and 6 were prepared from MM303, a multiply marked strain which may resolve plasmid oligomers less well than the prototrophic W3110, used to prepare the DNA in Lane 2.

The bands which decrease on ExoV treatment are not likely to be linear DNA, but rather super-coiled DNA which is nicked by contaminating nucleases when ExoV buffer is supplied.



Lane1: P1 linear DNA
Lane2: p^{pac}plasmid DNA
Lane3: p^{pac}plasmid DNA cut by BamHI
Lane4: p^{pac}plasmid DNA from recipients after transduction **(O/N cultured)**
Lane5: p^{pac}plasmid DNA from recipients after transduction cut by BamHI
Lane6: p^{pac}plasmid DNA from recipients after transduction + ExoV
Lane7: MBI 1KB DNA Ladder

Fig 5.2 Electrophoresis analysis of the plasmid DNA purified from P1 transductants selected on plates for AmpR. Lane 2: plasmid DNA purified from W3110. Lanes 4 and 6: Plasmid DNA prepared from MM303 transductants ExoV treatment was carried out as follows: p^{pac}purified from MM303 was mixed with ExoV, ATP and 10 x ExoV reaction buffer (see Materials and Methods for the recipe) and then digestion was carried out at 37°C for 2h.BamHI has a single cut on p*Pac* plasmid.

5.3. Discussion

The results presented in this section, combined with those of S. McAteer (Sean McAteer, personal communication), show that: 1) plasmids with homology to chromosomal DNA or P1 DNA can be transduced by P1 with high frequencies to *rec*$^+$ recipients, 100-1000 times higher than plasmids with no homology are. 2) the *rec* system is essential for plasmid establishment in recipients, since no transductants could be observed when a *rec*$^-$ strain is used as the recipient. 3) The site of P1 homology need not be the *pac* site.

Considering the small size of plasmids and the much larger content of the P1 heads, it is theoretically possible that the plasmids are packaged into P1 heads as passengers. If this was so, there is no reason why homology to either chromosomal or phage DNA should be necessary and transduction should also be *rec*-independent in the recipient cell. However, according to our results, the transduction process is *rec*-dependent in the recipient cells, irrespective of the homology to the chromosome or to the P1 genome and pBR325 alone is not transduced. By combining the results presented here with previous results, we can conclude that so long as homology to the P1 genome or the chromosome is present on plasmids, the transduction frequency of the plasmid by P1 is greatly increased, suggesting that recombination is responsible. On the other hand, it also indicates that plasmids cannot just be transduced by P1 simply as passengers.

There are in principle two ways in which plasmid DNA can become part of packagable concatemers. The first is by integrating plasmid DNA into either the chromosome or the P1 genome by recombination mechanisms. The second is by somehow creating concatemers of greater than 100KB that might be packageable into P1 heads. In the case of a plasmid with P1 homology, one could imagine this occurring if the plasmid pairs with P1 during the rolling circle phase of replication and somehow enters into rolling circle replication itself, to produce a concatemer of sufficient length. This seems less likely in the case of plasmids with chromosomal homology, since rolling circle replication is not characteristic of chromosome replication. It is perhaps possible, since spontaneous plasmid oligomers are common in the cell (see Fig 5.2), that such a structure is integrated into the chromosome by homology and then increased in length by replicational slippage or integration of further plasmid

monomers. This would presumably happen after P1 infection as hi-copy number plasmids are not ordinarily maintainable in the chromosome of growing cells. *Pac* containing strains could be used to ask whether plasmids with chromosome homology are packaged while integrated. If the homology on the plasmid is to a marker on the chromosome very close to a *pac* site such as *met* is, then we should be able to observe an increased number of transductants to ampicillin resistance, as compared to that of a *pac*-free strain.

We have already demonstrated that P1 plasmid transduction requires *RecA* in the recipient cell. This suggests that the *RecA* recombination system either plays a role in excising and recircularising the transduced plasmid DNA in the recipient cell or in converting plasmid oligomers back to monomers. To understand more about the form of plasmid precursors for packaging, it would be desirable to investigate whether or not the *rec* system is required in the donor cell. This is difficult to do because P1 produces very poor lysates from *recA* strains with a titer too low for successful transduction. However, according to preliminary work that I have done (data not shown), this problem could possibly be solved by making large-scale single cycle P1 lysates on *recA* strains, instead of making overnight plate lysates.

Of course, it is also very reasonable that P1 plasmid transduction may involve more than one mechanism. Both of the above mechanisms may be working at the same time. Knowledge about plasmid transduction by P1 is still far from complete.

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